

Transplantation of endothelial progenitor cells derived exosomes improve angiogenesis in a rat hindlimb ischemia model

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Research Article

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Abstract

Objective

We investigated whether endothelial progenitor cells (EPCs) derived exosomes (EPC-exo) could improve angiogenesis in a rat hindlimb ischemia model to evaluate whether the EPC-exo may represent a potential therapeutic strategy for lower limb ischemia (LLI).

Methods

EPCs were obtained from SD rats using the method of density gradient centrifugation, and identified by DIL-AC-LDL and FITC-UEA-I uptake test and flow cytometry. EPC-exo was isolated and purified from EPCs using ultracentrifugation method, and identified by transmission electron microscopy and western blot analysis. Rat chronic hindlimb ischemia model were divided randomly into three groups, the experimental group (n=5) was intramuscularly injected with 30ul EPC-exo through four points in ischemic hindlimb, while the EPC group (n=5) was injected with 30ul EPCs, and the control group (n=5) was injected with 30ul PBS. Laser doppler perfusion imaging (LDPI) was used to assessment blood flow perfusion of rat hindlimb at baseline, 7 day, 28 day after intramuscularly injection. Capillary density of muscle tissue was evaluated by immunofluorescence analysis at 28 day. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was used to detect the expression change of vascular related genes in endothelial cells (ECs) induced by EPC-exo.

Results

DIL-AC-LDL and FITC-UEA-I uptake test showed that more than 90% of the EPCs were double-positive. Flow cytometry showed that in our cultured EPCs, the positive rates of CD31, CD34, CD133 and VEGFR-2 were 97.8%, 85.3%, 83.2% and 91.3%, respectively, but the positive rate of CD45 was only 1.3%. EPC-exo with a size ranging from 30 to 100 nm and western blot analysis confirmed the presence of exosomes markers, including CD9, CD63 and CD81. Compared with non ischemic limbs, EPC-exo group recovered about 80% of the blood perfusion after 28 days, while the EPC group had a recovery rate of about 60%, and the control group had a recovery rate of about 40% only. Capillary density of muscle tissue in EPC-exo group increased significantly than EPC group and control group. qRT-PCR detected the expression of vascular related genes (VEGFA, VEGFR2, HIF-1a, eNOS, ANG1, ANG2, CXCL-16) increased significantly in EPC-exo group than control group.

Conclusion

The study results suggest that transplantation of EPC-exo could significantly improve angiogenesis in a rat hindlimb ischemia model, which may be a potential therapeutic strategy for LLI.

Background

Lower limb ischemia (LLI) is mainly caused by arteriosclerosis obliterans (ASO) and the morbidity is more than 12% in over 60 years general population^[1]. The typical clinical manifestation is intermittent claudication and limb gangrene entails a high risk of limb amputation. Surgical and endovascular revascularization are the main treatment methods for LLI. However, many patients can not accept surgical or endovascular revascularization because of limited vascular condition and general condition. Therapeutic neovascularization maybe is the last straw for these patients^[2].

At present, it is believed that neovascularization usually occurs through two mechanisms, angiogenesis and vasculogenesis^[3]. In 1997, Asahara et al found that there are precursor cells in the circulating peripheral blood which can differentiate into vascular endothelial cells (ECs) after birth for the first time, and called the cells as EPCs^[4]. Subsequently, a large number of evidences have shown that there is not only angiogenesis after birth, but also postnatal vasculogenesis dependent on EPCs^[5].

However, some recent studies have reported that the neovascularization of EPCs was not directly rely on differentiate into ECs but by paracrine mechanism^[6-7]. Exosomes play an important role in paracrine mechanism, which is a lipid bilayer extracellular vesicle with a diameter of 30-100nm^[8-10]. Exosomes contain a variety of functional proteins, lipids, messenger RNA (mRNA), and microRNA (miRNA). Exosomes can not only protect their contents from enzymatic degradation through their lipid bilayer structure, but also transport them through various body fluids including blood. Therefore, exosomes are very important for intercellular communication^[11].

In the present study we investigated whether EPCs derived exosomes (EPC-exo) could improve angiogenesis in a rat hindlimb ischemia model to evaluate whether the EPC-exo may represent a potential therapeutic strategy for LLI.

Materials And Methods

Animals

All animal experimental protocols in this study were reviewed and approved by the Institute of Animal Care and Use Committee of Capital Medical University Affiliated Beijing Chaoyang Hospital. All animal procedures were performed according to NIH guidelines for the care and use of laboratory animals. The animal were maintained under a conventional state and fed with standard laboratory food and water. The study was conducted in accordance with the ethical principle of the World Medical Association Declaration of Helsinki, and local legislation. This study was carried out in compliance with the ARRIVE guidelines.

Rat bone marrow derived-EPCs cultures

Rat bone marrow mononuclear cells (BM-MNCs) were collected and isolated as previously reported^[12]. Briefly, after centrifugation, the cells were divided into four layers. The second layer was MNCs. The

MNCs were suspended in EGM-2MV medium (containing 20% fetal bovine serum, vascular endothelial growth factor, recombinant basic fibroblast growth factor, insulin-like growth factor, recombinant epidermal growth factor, hydrocortisone and ascorbic acid, etc.) and inoculated with $1 \times 10^6/\text{cm}^2$ in a 25 cm^2 culture bottle with FN ($2 \mu\text{g}/\text{cm}^2$). Then the cells were cultured in a 5% CO₂ saturated humidity cell incubator at 37 °C. When cells fusion over 80%, cells were harvested with 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) and cultured passage continuously. Cells growth was observed by fluorescent microscope every day.

Identification of EPCs

EPCs were identified by DIL-AC-LDL and FITC-UEA-I uptake test and flow cytometry. Flow cytometry was used to identify the expression of CD31, CD34, CD133, VEGFR-2 and CD45 on EPCs surface.

The primary passage EPCs were digested with trypsin and inoculated into a 12 well plate, after cells adhered to the wall, DIL-AC-LDL ($10 \mu\text{g}/\text{ml}$; Molecular Probes, Carlsbad, Calif) and FITC-UEA-I ($10 \mu\text{g}/\text{ml}$; Sigma) was added into each well plate and incubated in 5% CO₂ incubator at 37 °C for 4 hours.

After EPCs fusion over 90%, 0.25% trypsin was performed to digest the cells. $100 \mu\text{l}$ of FACS buffer solution suspended 5×10^5 Of EPCs, add different fluorescent labeled monoclonal antibodies (FITC labeled mouse anti human CD31, CD45, PE labeled mouse anti human CD133, VEGFR-2 APC labeled mouse anti human CD34) and well mixed, incubation at 4°C for 15 minutes, and then detected by flow cytometry (FACScan, Becton Dickinson).

Purification of EPC-exo

2-3 passages EPCs fusion over 80%, EGM-2 MV medium was washed with PBS for 3 times, and new EGM-2 MV medium replaced and cultured go on for 48 hours under hypoxic conditions. The supernatants were collected and centrifuged at $300 \times g$ for 5 minutes at 4°C, then the precipitate was discarded, and centrifuged at $1000 \times g$ for 15 minutes at 4°C to remove cell debris, and then filtered through a $0.22 \mu\text{m}$ filter.

The supernatants were transferred to the ultrafiltration tube and centrifuged at $10,000 \times g$ at 4°C for 1 hour. Subsequently, the remaining supernatant was purified after 2 centrifugations at $100,000 \times g$ at 4 °C for 2 hours at 4 °C. The pellets were resuspended with PBS and stored at -80°C until use. The morphology of exosomes were observed by transmission electron microscopy.

Western blot analysis

Western blot analysis was used to identify the following surface markers of EPC-exo: CD9, CD63 and CD81. Protein extracted from the exosomes samples was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), and then incubated with CD9, CD63 and CD81 antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies.

Rat chronic hindlimb ischemia model and intramuscularly injection

3 months old SD rats, weighting 175-210g, were anesthetized by inhalation of 2% isoflurane. Under sterile conditions, a 1.5cm skin incision was made overlying the middle portion of the left hindlimb of each rat, and was pulled away with a small retractor. The adipose tissue around thigh muscle was pushed with cotton swab to expose the vascular and nerve bundles including femoral artery, femoral vein and femoral nerve. The branches of superficial circumflex iliac artery, deep femoral artery, abdominal pudendal artery and descending genicular artery were ligated with 7-0 silk suture. After temporary blocked with vascular clamp, a small transverse incision was made in the anterior wall of femoral artery. A silicone tube was inserted into the femoral artery through the small transverse incision and fixed with 7-0 silk suture. The process of chronic thrombosis in silicone tube was used to simulate the pathology of peripheral artery occlusion.

7 days later after the surgery, the rats were divided randomly into three groups. The experimental group (n=5) was intramuscularly injected with 30ul EPC-exo through four points in ischemic hindlimb, while the EPC group (n=5) was injected with 30ul EPCs, and the control group (n=5) was injected with 30ul PBS.

Laser doppler perfusion imaging (LDPI)

Laser doppler perfusion imaging (LDPI) (Moor Instruments, Devon, UK) , as a non-invasive measurement, used to assessment blood flow perfusion of rat hindlimb. Rats were anesthetized with 2% isoflurane and subjected to LDPI measurement at baseline, 7 day, 28 day after intramuscularly injection. At each time, both ischemic limb and non-ischemic limb should be measured simultaneously. The images were analyzed to compare the blood flow ratio of the ischemic limb vs. the non-ischemic limb expressed as percentage perfusion.

Immunofluorescence analysis evaluation of capillary density

Capillary density of muscle tissue was evaluated by immunofluorescence analysis, 28 day after intramuscularly injection, rats were sacrificed to harvest muscle tissue. Muscle samples were fixed in methanol, paraffin embedded, and cross-sectioned (5 μ m), and monoclonal antibody against mouse CD31 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used. The number of CD-31-positive per high power field (x400) was represents capillary density.

Quantitative reverse-transcriptase polymerase chain reaction

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was used to detect the expression change of vascular related genes in ECs induced by EPC-exo. 5×10^5 ECs were seeded in a six well plate, when the cell adhesion and fusion degree was about 80%, the cells culture medium was changed. The experimental group (containing 100ug/mL EPC-exo), the EPC group (containing 100ug/mL EPCs) and the control group (containing 100ug/mL PBS), were cultured in the cell incubator with 5% CO₂ at 37°C for 24 hours.

Total RNA was extracted with Trizol reagent (Invitrogen) following the manufacturer's protocol, and cDNA was synthesized from 1 µg of extracted total RNA using the PrimeScript RT reagent kit (TAKARA, Tokyo, Japan). Then, 1 µl of cDNA sample was used as a template for qRT-PCR using an ABI PRISM 7900HT System with SYBR Premix ExTaq II (Takara Biotechnology). The following human primers were used: VEGFA, VEGFR2, HIF-1a, eNOS, ANG1, ANG2, CXCL-16. The primer sequences used in the study are summarized in Table 1. Relative gene expression data were analyzed with the $2^{-\Delta\Delta C_t}$ method.

Statistical Analysis

The data were shown as the means \pm SEM and analyzed using GraphPad Prism 7.0 software. Unpaired Student's t test was used for statistical comparison of the data. Differences between groups were considered statistically significant at $p < 0.05$.

Results

EPCs cultures

Rat EPCs were isolated and purified by density centrifugation and the morphology of EPCs were observed by fluorescent microscope. After 48 hours of culture, the adherent cells grew like spindle shaped, triangular, spindle shaped or irregular (Fig 1). After 7 days of culture, the cells grew radially around the colony gradually arranged into monolayer (Fig 2); After 14-21 days of culture, all the colonies fused, and the cells were in the shape of cobblestone (Fig. 3). The primary passage cells take about 25-30 days. After the second-third passage, the cells were obviously aging and their vitality is reduced. We digested them at the 7th day for identification.

EPCs identification

The light staining results showed that the cells cytoplasm absorption of DIL-AC-LDL presented red fluorescence (Fig. 4), the cells cytoplasm absorption of FITC-UEA-1 presented green fluorescence (Fig. 5), and the double-stained positive cells presented yellow fluorescence (Fig. 6). In this experiment, it was found that more than 90% of the cells were double-positive.

Flow cytometry was used to detect CD31, CD34, CD133, VEGFR-2 and CD45 expression on the surface of P2-P3 cells. The positive rates of CD31, CD34, CD133 and VEGFR-2 were 97.8%, 85.3%, 83.2% and 91.3% respectively, but the positive rate of CD45 was only 1.3% (Fig. 7), so, it can be considered that high purity EPCs were obtained in the study.

Characterization of EPC-exo

EPC-exo was identified by transmission electron microscopy and western blot analysis, Morphological analysis of EPC-Exo using transmission electron microscopy demonstrated the typical appearance of microvesicles, with a size ranging from 30 to 100 nm (Fig. 8). Western blot analysis confirmed the presence of exosomes markers, including CD9, CD63 and CD81 (Fig. 9).

Laser doppler perfusion imaging (LDPI)

We established the rat hindlimb ischemia model by inserting anticoagulant silicone tube into femoral artery, and then injected EPC-exo, EPC or PBS intramuscularly. The results showed that compared with non ischemic limbs, EPC-exo group recovered about 80% of the blood perfusion after 28 days, while the EPC group had a recovery rate of about 60%, and the control group had a recovery rate of about 40% only (Fig.10,11). It is suggested that EPC-exo transplantation could significantly improve the limb blood flow perfusion recovery in rat hindlimb ischemia model.

Evaluation of capillary density

CD31 positive staining was brown, mainly expressed in intercellular and paramuscular ECs, quantitative analysis of CD31 positive cells showed that, compared with PBS control group and EPC group, the expression of CD31 positive in EPC-exo group was significantly increased, and the difference was statistically significant ($P < 0.01$ vs. control group and $P < 0.05$ vs. EPC group) (Fig.12).

Quantitative reverse-transcriptase polymerase chain reaction

The expression of vascular related genes (VEGFA, VEGFR2, HIF-1a, eNOS, ANG1, ANG2, CXCL-16) in ECs increased significantly in EPC-exo group than control group. ($P < 0.05$) (Fig.13).

Discussion

LLI refers to lower limbs insufficient blood perfusion caused by various reasons, resulting in intermittent claudication, skin ulcers, and even gangrene and amputation^[13]. Currently, the main treatment option of LLI is surgical or endovascular revascularization to restore the limb blood perfusion, but the results were often not satisfactory and many patients could not accept the invasive therapy^[14]. In recent years, therapeutic angiogenesis technology based on stem cell transplantation was a worthwhile method to LLI patients especially which could not accept surgical or endovascular revascularization^[15].

EPCs are adult stem cells with the ability of self renewal and differentiation into mature ECs. In 1997, Asahara, a Japanese scientist, the first time reported a class of CD34 + cells in human peripheral blood, which can differentiate into mature vascular ECs under the condition of specific growth factor medium, and defined them as EPCs^[4]. In the early stage, EPCs were thought to be involved in the process of therapeutic angiogenesis by directly differentiating into mature vascular ECs at the damaged intima^[16]. In recent years, many studies have shown that EPCs do not directly differentiate into mature ECs, but promoting the proliferation and migration of the original residual ECs through secreting a variety of cytokines. Exosomes, as a major form of paracrine cytokines, has been confirmed as an important intercellular communication mediator in both physiological and pathological processes^[17-18]. Exosomes may bind to the target cell membrane to obtain new surface molecules and new adhesion properties. In addition, they can exchange proteins in cell membrane and cytoplasm between cells^[19].

EPCs transplantation have been used in many animal experiments to treat hindlimb ischemia model and have achieved certain satisfactory results^[20-21]. However, EPCs transplantation could be used in clinical treatment of ischemic diseases, there are still many problems that need to be solved. Firstly, many risk factors can reduce the number and function of circulating EPCs, such as old age, smoking, diabetes, so in vitro amplification is an inevitable way^[22]. Secondly, EPCs in the long-term in vitro culture environment could lead to telomerase degradation, gene and epigenetic changes. When in vitro cultured EPCs transplanted into the human body, there was a potential risk of tumor formation^[23]. Lastly, the low survival rate after EPCs transplantation had not been solved satisfactorily due to the influence of the health status of the body and the site of sampling^[24].

Many studies have confirmed the effectiveness of exosomes in treatment of ischemic diseases. Living cells contain extracellular vesicles (EVs) and other components. EVs consist of three major parts including exosomes, microvesicles (MVs) and apoptotic bodies^[25]. Ibrahim et al found that exosomes released by human cardiosphere-derived cells (CDCs) inhibit apoptosis of cardiomyocytes and promote angiogenesis in a mouse myocardial ischemic model. The findings revealed the potential utility of exosomes as cell-free therapeutic candidates^[26]. Zhao Y et al intravenously human umbilical cord mesenchymal stem cell (MSC) derived exosomes into left anterior descending coronary artery in rats, the study found that the exosomes have a protective effect on acute myocardial infarction (AMI)^[27]. Some studies have found that the levels of circulating exosomes miR-126, miR-9, miR-124 and miR-223 increase when cerebral ischemia occurs, and the more severe the ischemia, the higher the miR levels^[28-29]. Doeppner et al found that systemic administration of MSC-derived extracellular vesicles (EVs) remarkably promotes neurovascular remodeling and functional recovery in a C57BL6 mice cerebral ischemia model. MSC-EVs have been shown to be safe in humans, the study provides relevant clinical evidence warranting rapid proof-of-concept studies in cerebral ischemia patients^[30]. Wang J et al found that EPC-derived microvesicles (EPC-MVs) protect human brain microvascular ECs (hb-ECs) from hypoxia/reoxygenation (H/R) injury, which were associated with the changes of EPC-MVs carried miR126 and eNOS to control the PI3K/eNOS/NO pathway^[31].

Recently, more and more studies had explored the function of exosomes in treatment of hindlimb ischemia disease. In 2012, Ranghino et al randomly divided the SCID mice hindlimb ischemia model into 4 groups, then treated with EPC-derived MVs, RNase-inactivated MVs, fibroblast derived MVs or vehicle alone (control group) respectively. The study found that limb perfusion of MVs group significantly higher than control group^[32]. Hu GW et al randomly divided the mouse hindlimb ischemia model into 2 groups, then treated with multiple intramuscular injections in the ischemic leg with exosomes derived MSCs (MSCs-exo) or PBS (control group) respectively. The study found that MSCs-Exo markedly enhanced microvessel density and blood perfusion than control group^[33].

Despite exosomes' seemingly homogenous characteristics including size and surface markers, isolation and purification of exosomes is not an easy work. Different methods have been developed to extract exosomes, one of the most common methods was used centrifugation to remove debris and dead cells,

and then used high-speed centrifugation to extract exosomes, as described in our study. We observed that the exosomes derived from EPCs were round or oval, and the diameter was about 30-100nm. Western blot analysis confirmed that EPC-exo expressed CD9, CD63 and CD81. CD9, CD63 and CD81 belongs to the transmembrane 4 family/tetra-spanins (tm4sf / tspan), which are special cell membrane glycoproteins. CD9, CD63 and CD81 are related to cell fusion and play an important role in monocyte fusion^[34]. Combined use of CD9, CD63 and CD81 can significantly increase the migration rate of dendritic cells, and reduce the expression of integrin in the extracellular matrix^[35].

In the study we observed that EPC-exo transplantation improve angiogenesis in a rat hindlimb ischemia model superior to EPC and PBS. LDPI and capillary density evaluation results suggested that EPC-exo transplantation can significantly improve the limb blood flow perfusion recovery in ischemic hindlimb. The function of ECs, such as proliferation, migration, and tube formation ability, is closely related to the expression levels of various angiogenesis related genes. Our qRT-PCR results showed that after 24 hours culture with EPC-exo, a variety of angiogenesis genes (VEGFA, VEGFR2, HIF-1a, eNOS, ANG1, ANG2, CXCL-16) were detected increased significantly. Our results suggested that EPC-exo have a strong effect in promoting the proliferation and migration of vascular ECs.

In our study, we used EPC-exo transplantation, which could be used more safely compared to the EPCs transplantation. The EPC-exo have certain functions of EPCs, and have no oncogenic harm, so could avoid the potential risk of direct transplantation of EPCs. It is an ideal method for therapeutic angiogenesis. In summary, we have shown that EPC-exo have the therapeutic angiogenesis potential in a rat hindlimb ischemia model.

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Table

Table.1. 1 Primer sequences for quantitative reverse-transcriptase polymerase chain reaction

Gene	Forward primer (5'-3')	Reverse Primer (5'-3')
VEGFA	CGCTCGGTGCTGGAATTTGA	AGTGGGGAATGGCAAGCAAA
VEGFR2	GGAAATGACCTTCATCCCTACC	GCTGACCAAGACGGTTGTATCT
HIF-1a	CCCTGAAGATTCCTGAACGAG	CCATAGGCAAAAGCAGTGAGC
eNOS	GCTACGAGGAGTGAAGTGGT	CAGCTACAGTGAGGTGGATCTCTC
ANG1	AGGGAACCGAGCCTATTCACAG	CCACAAGCATCAAACCACCATC
ANG2	ATCTTGATAAC CGCAGCCAC	TGTCGGCACA TACCTCTTGT
CXCL-16	GCAGCGTCACTGGAAGTTGTT	AGCTCATCAATTCCTGAACCCA

Figures

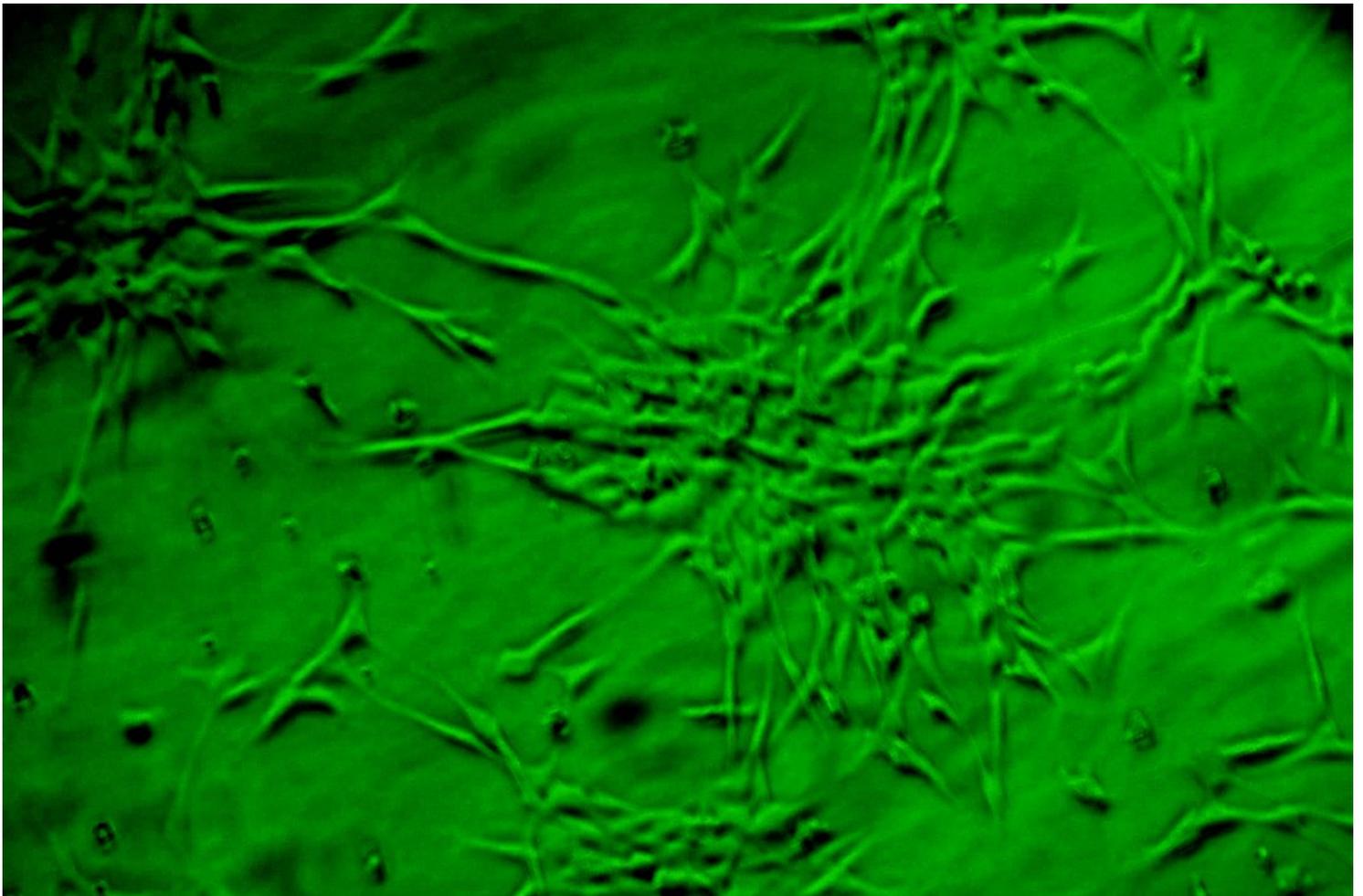


Figure 1

After 48 hours of culture, the adherent cells grew like spindle shaped, triangular, spindle shaped or irregular.

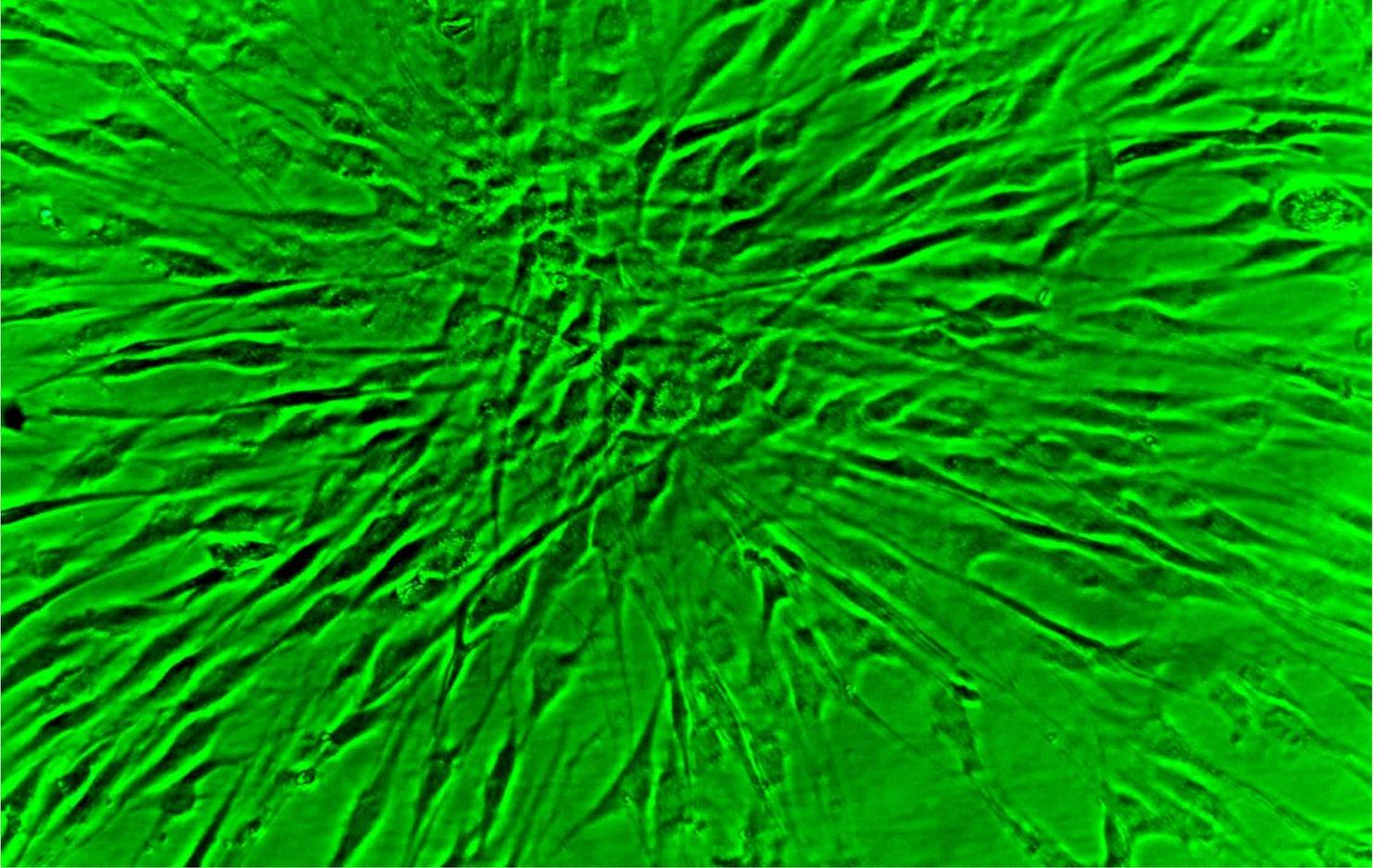


Figure 2

After 7 days of culture, the cells grew radially around the colony gradually arranged into monolayer.

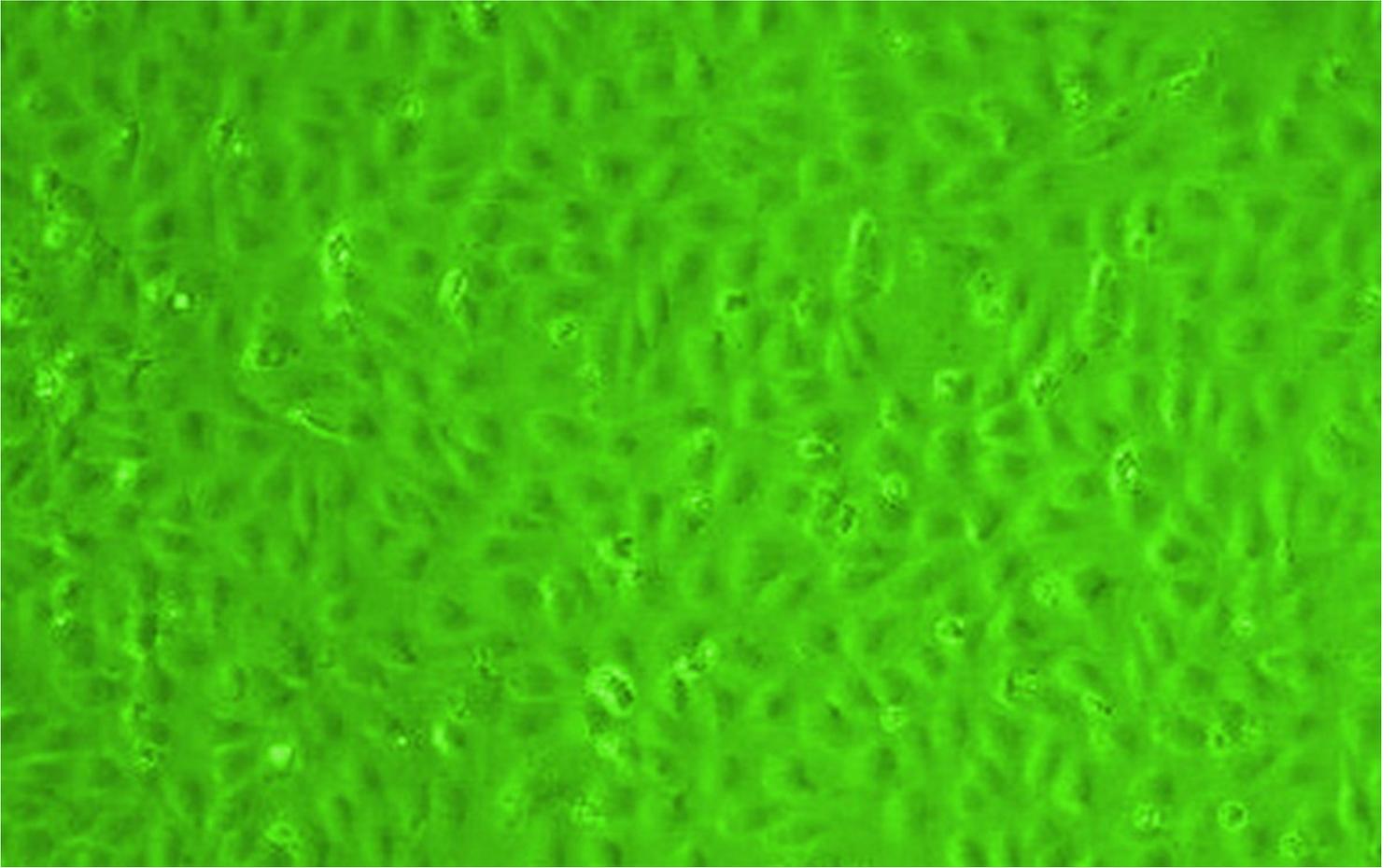


Figure 3

After 14-21 days of culture, all the colonies fused, and the cells were in the shape of cobblestone.

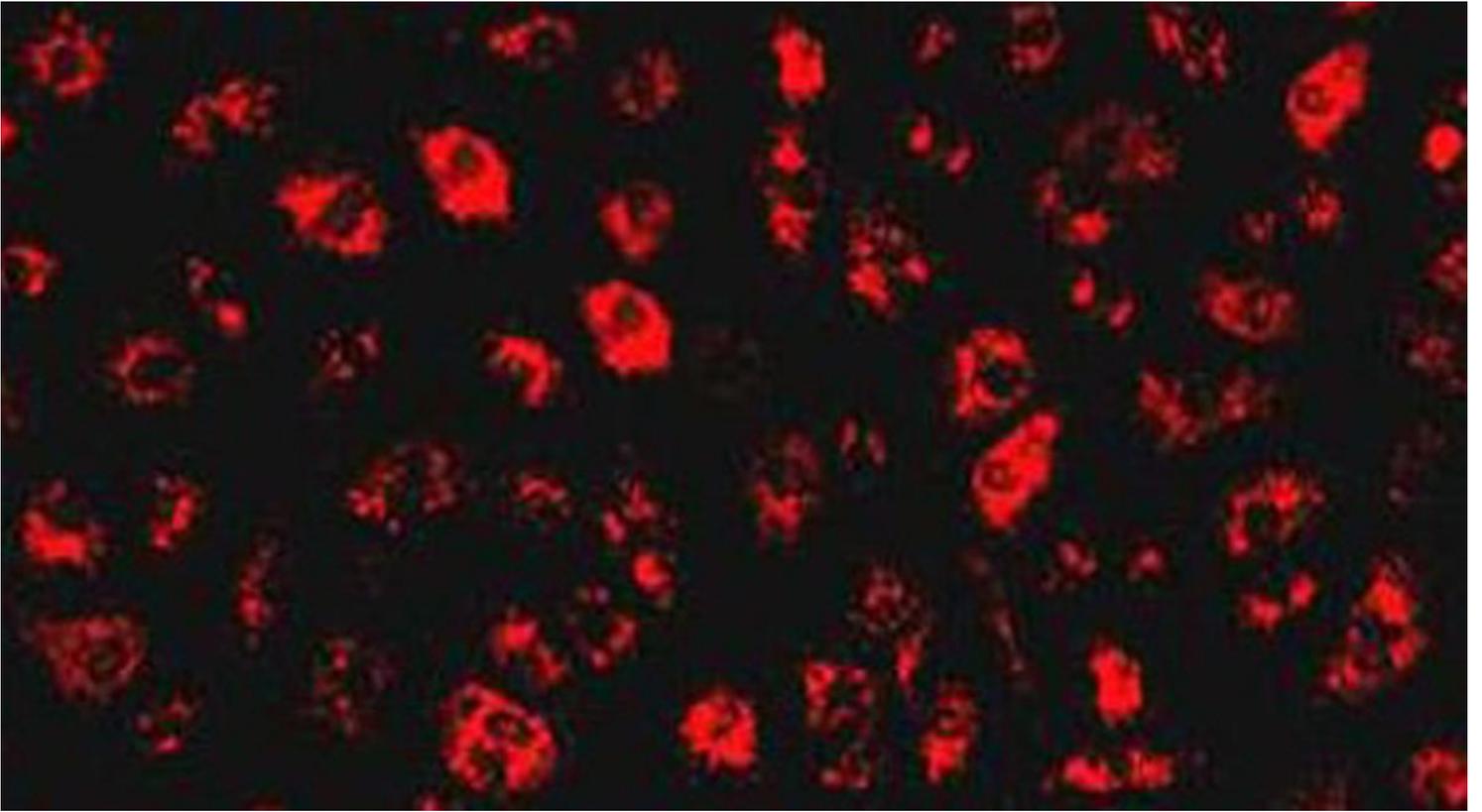


Figure 4

EPC uptake Dil-acLDL test (red).

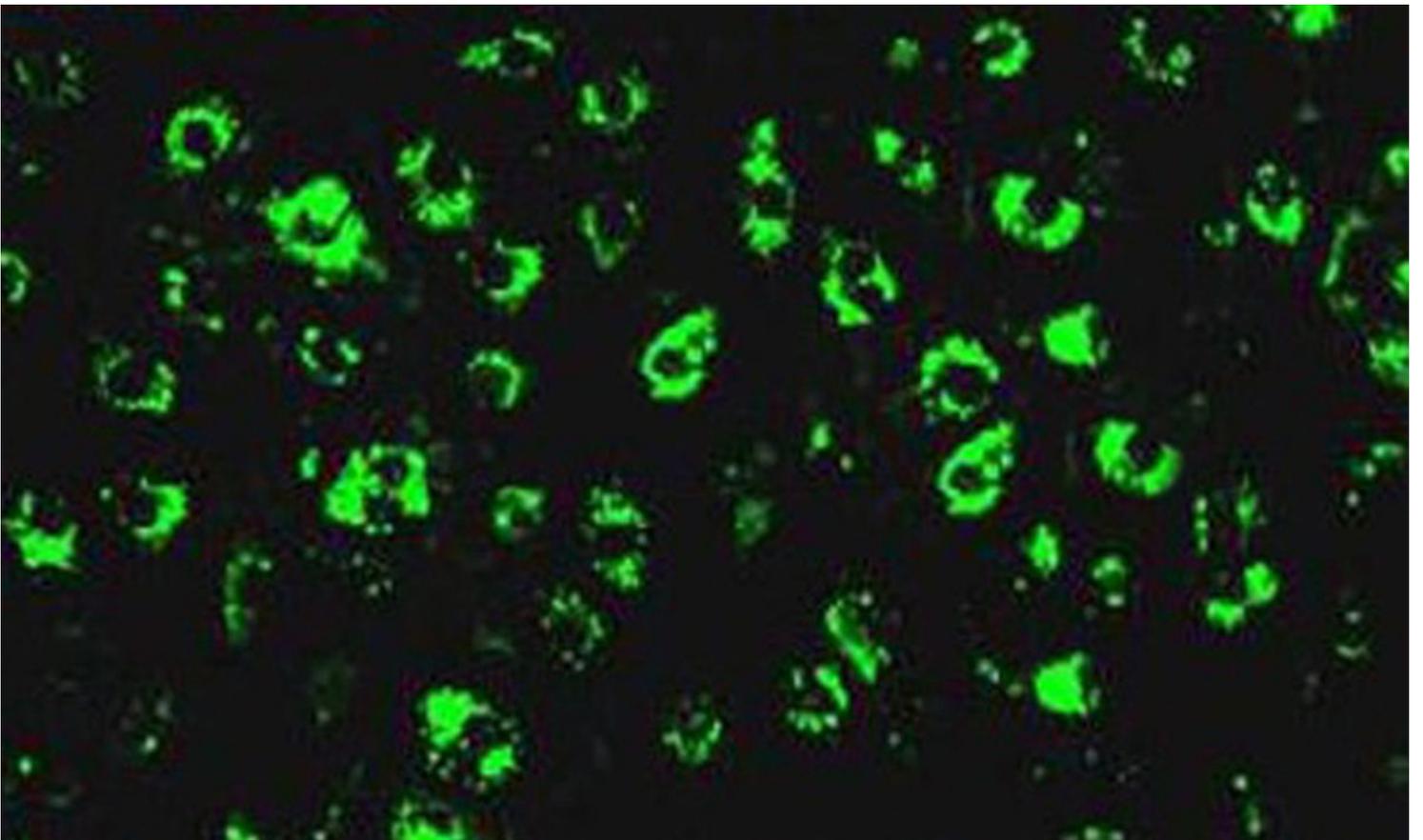


Figure 5

EPC uptake FITC-UEA-1 test (green).

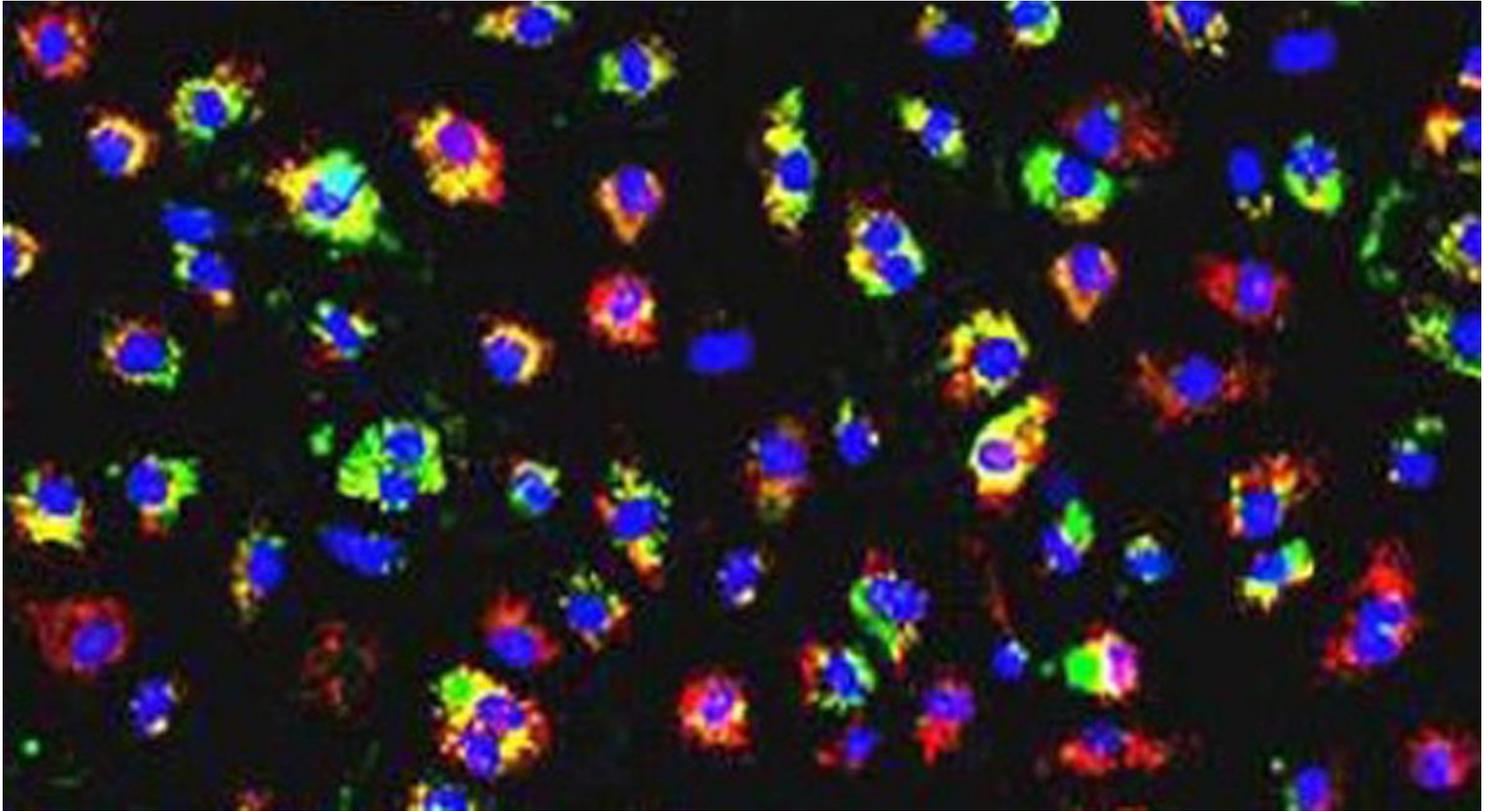


Figure 6

EPC uptake Dil-acLDL and FITC-UEA-1 test (yellow).

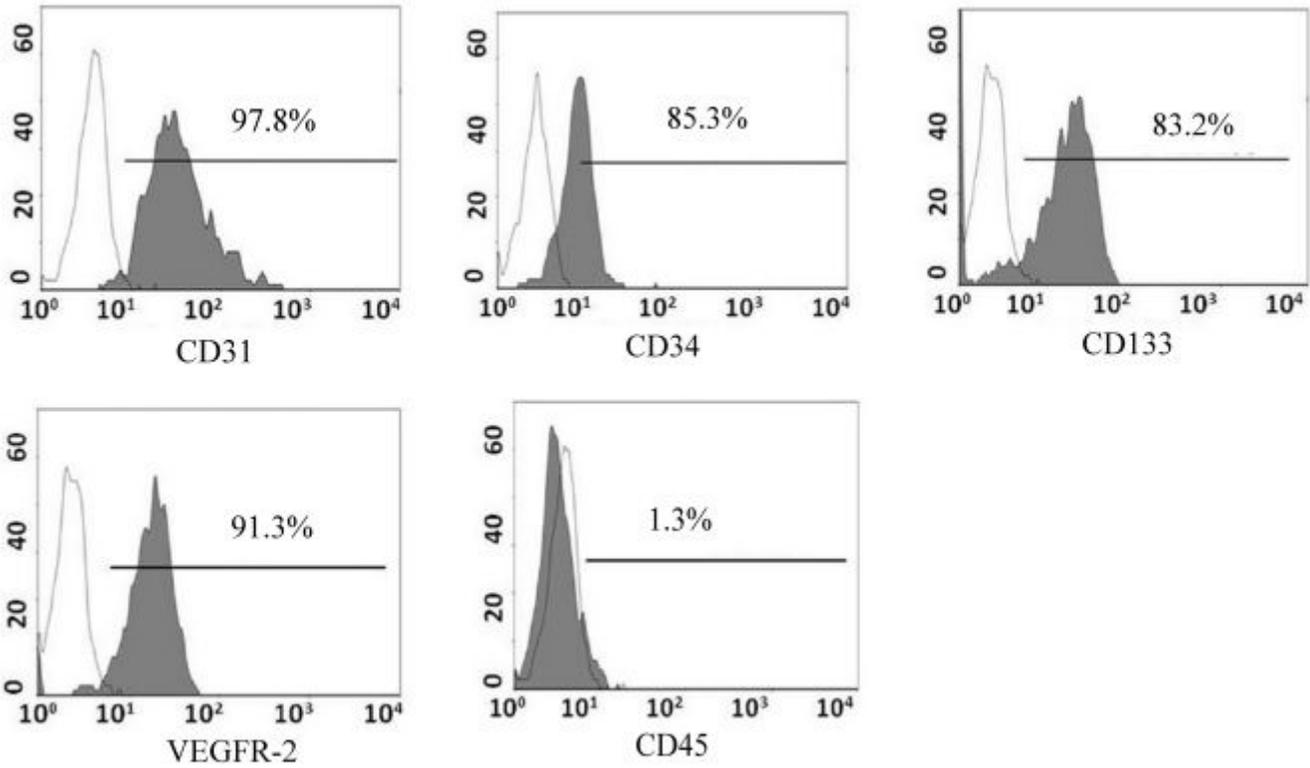


Figure 7

Flow cytometry was used to detect CD31, CD34, CD133, VEGFR-2 and CD45 expression on the surface of P2-P3 cells. The positive rates of CD31, CD34, CD133 and VEGFR-2 were 97.8%, 85.3%, 83.2% and 91.3%, respectively, but the positive rate of CD45 was only 1.3%, so, it can be considered that high purity EPCs were obtained in the study.

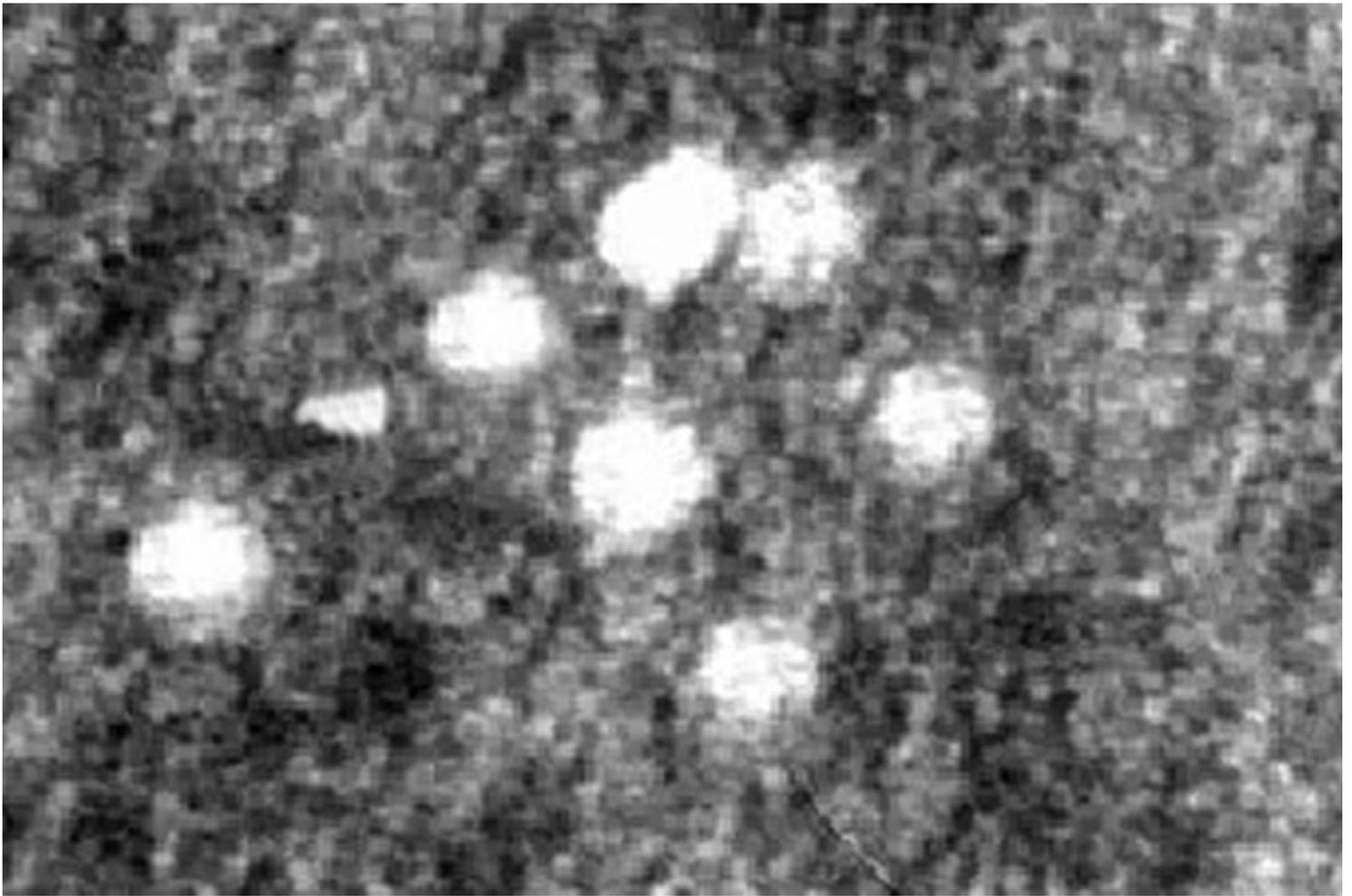


Figure 8

EPC-exo morphology under a transmission electron microscopy. Scale bar: 100 nm.

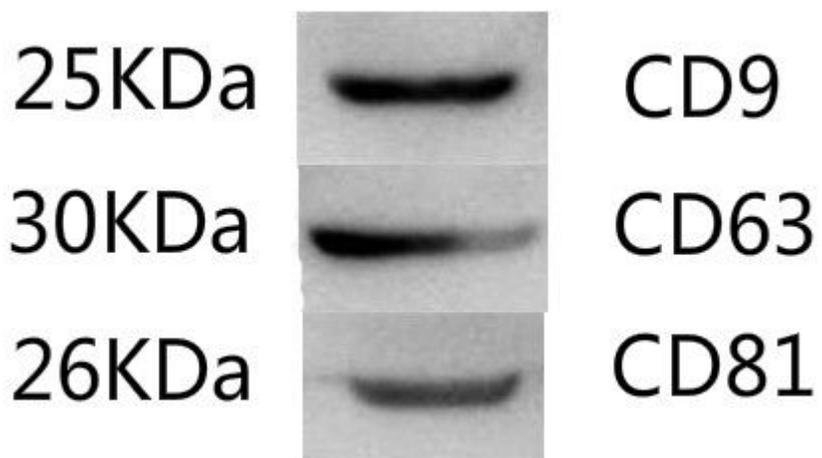


Figure 9

Western blot analysis of EPC-Exo surface marker CD9, CD63, and CD81.

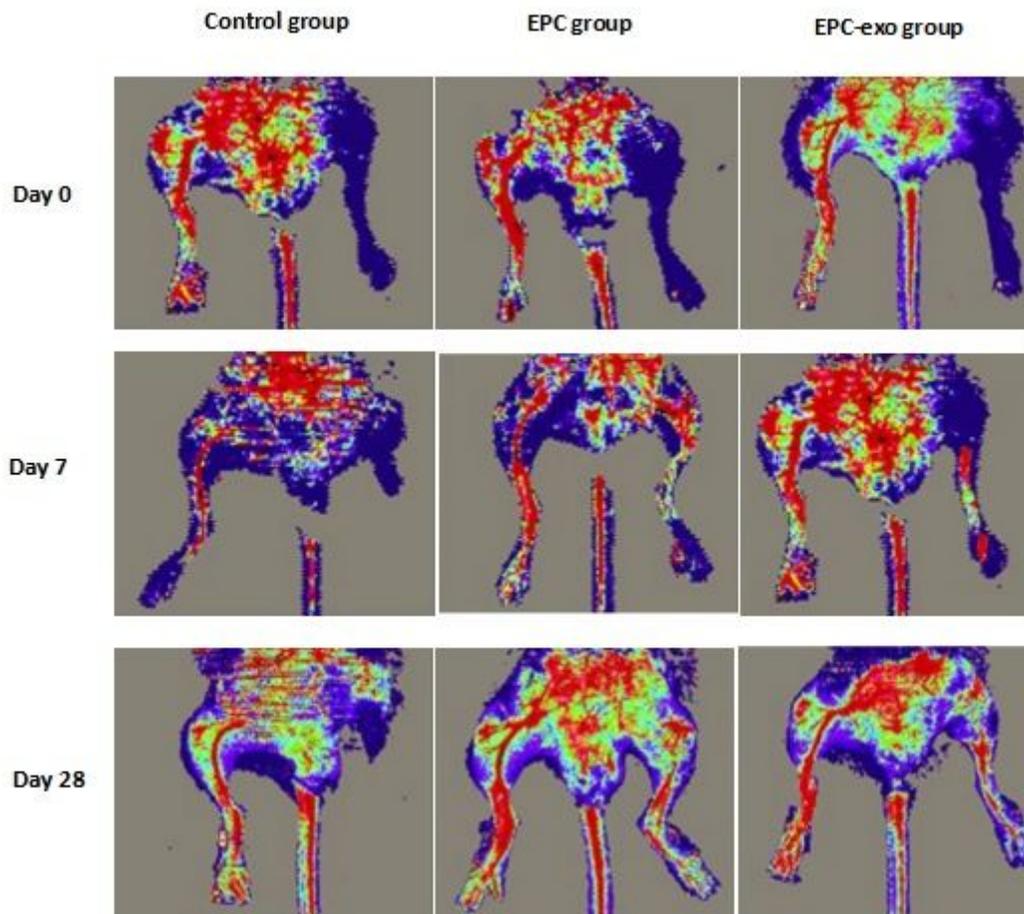


Figure 10

Laser doppler perfusion images (LDPI) of perfusion in rat ischemic hindlimb at 0 day, 7 day, and 28 day after intramuscularly injection with PBS, EPC, and EPC-exo.

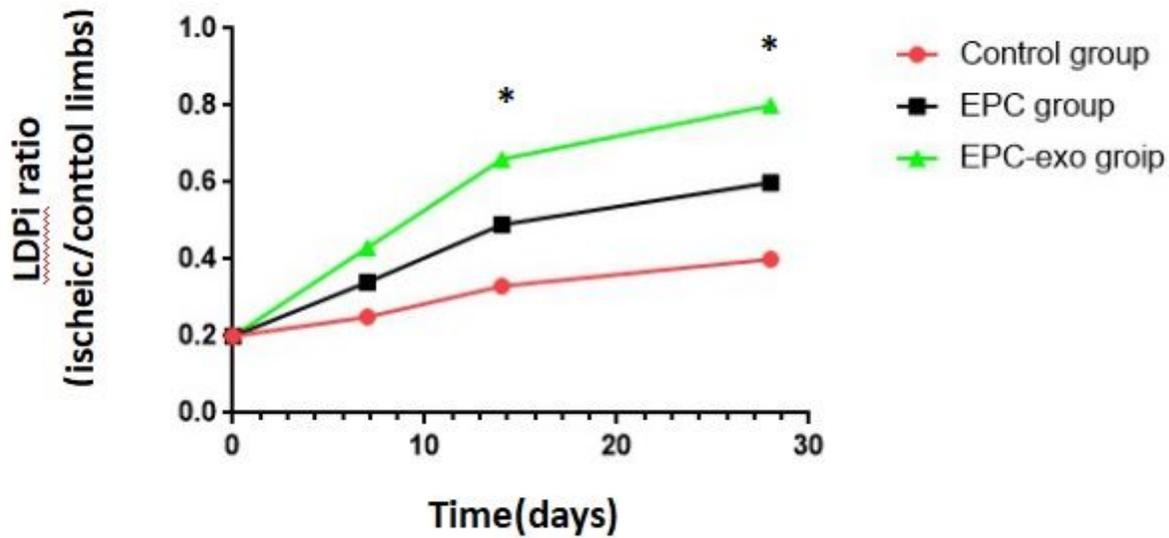


Figure 11

Laser doppler perfusion images (LDPI) measurements of perfusion ratio (Ischemic limb: no-ischemic limb) at 0 day, 7 day, 14 day, and 28 day after treatment (* $P < 0.05$, $n = 5$).

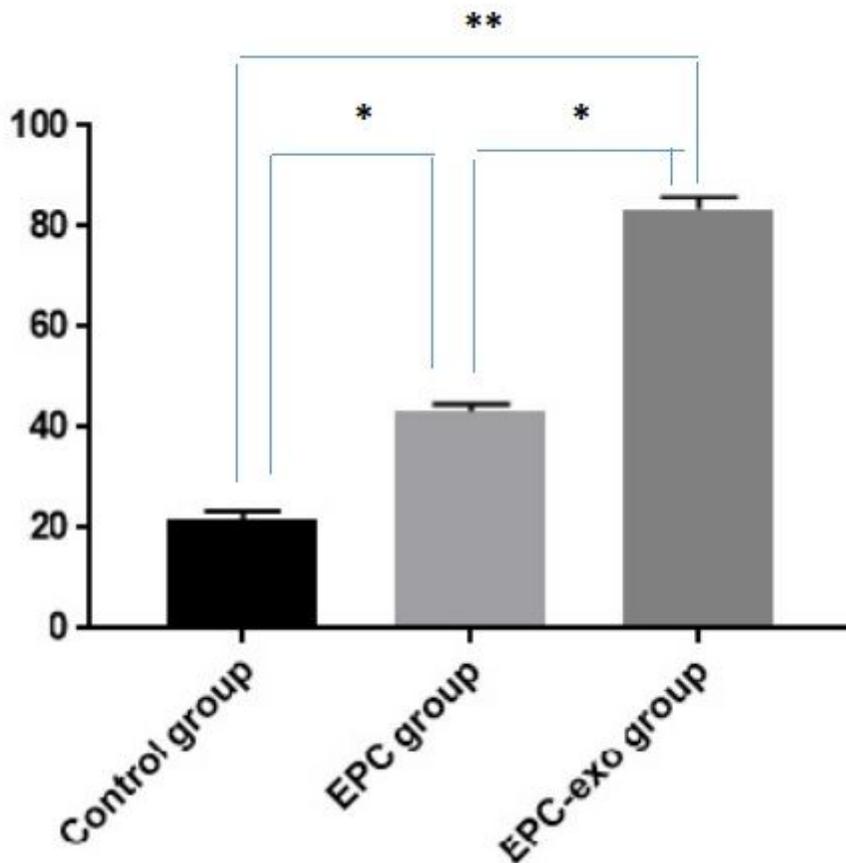


Figure 12

Quantification of capillary density in the ischemic limbs (**P<0.01 compared with control group, *P<0.05, compared with EPC group n = 5).

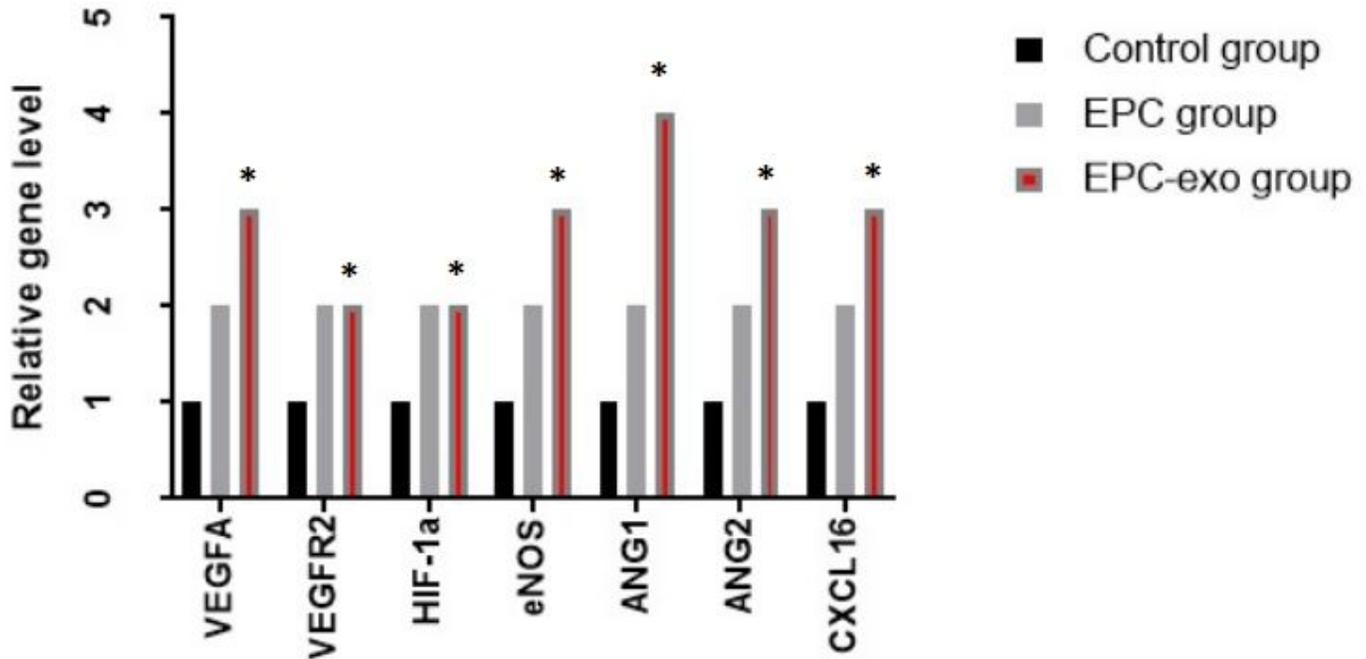


Figure 13

qRT-PCR was used to detect the expression of vascular related genes in ECs, and the data were analyzed with the $2^{-\Delta\Delta C_t}$ method. VEGFA, VEGFR2, HIF-1a, eNOS, ANG1, ANG2, CXCL-16 increased exponentially in EPC-exo group, which were significantly different from that in control group (P < 0.05).