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# Combination of cells-based therapy with apelin-13 and hyperbaric oxygen efficiently promote neovascularization in ischemic animal model

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**Abstract. – OBJECTIVE: Critical lower-limb ischemia (CLLI) is characterized by high morbidity and mortality. The aim of this study was to explore the effectiveness of the combination of cell therapy with apelin-13 and hyperbaric oxygen in CLLI animal model.**

**MATERIALS AND METHODS: The experimental ischemic rats were divided into five groups, including negative control, bone marrow derived mononuclear cells (BM-MNCs), apelin-13, hyperbaric oxygen treatment (HBOT) and apelin-13 with HBOT group. Each group was composed of 10 rats. Endothelial progenitor cells (EPCs) derived from bone marrow were transplanted into the ischemia rat model. After 3 weeks of transplantation, the formation of new vessels was evaluated by examining cluster of differentiation (CD)31, CD34 and vascular endothelial growth factor receptor 2 (VEGFR-2) expressions as well as a direct vision of vessels by hematoxylin and eosin (HE) staining and immunohistochemistry.**

**RESULTS: Compared with the negative control group, both angiogenic factors expressions and the number of new vessels increased notably by the transplantation of BM-MNCs in the ischemic models. Apelin-13 or HBOT alone improved the efficacy within limit while the combination of the three elements remarkably promoted the neovascularization in ischemic limbs.**

**CONCLUSIONS: BM-MNC induced angiogenesis in the ischemic limbs and was considered an effective resource for cell therapy. The preliminary data of this study showed that the combination of cell therapy with apelin-13 and HBOT improved the efficacy of angiogenesis.**

*Key Words:*
Neovascularization, Cell therapy, Apelin-13, Hyperbaric oxygen treatment.

## Introduction

Peripheral artery disease (PAD) is a cardiovascular condition characterized by the narrowing of the arteries due to the accumulation of atherosclerotic plaque[1]. In 2010, it was estimated that about 202 million people worldwide suffer from PAD[2]. With the aging of the world and the popularity of other risk factors such as smoking, the prevalence of PAD is expected to increase greatly in the near future[3]. The end stage of PAD, critical lower-limb ischemia (CLLI), is the most severe type associated with impaired quality of life and high risk of amputation or even mortality with dismal prognosis[4].

The gold standard in treatment for CLLI is the revascularization achieved by endovascular or surgical bypass[5], however about 20%-30% of patients did not respond for such therapies and therefore amputation is the last option[6]. A novel therapeutics cell-based therapy, achieved by delivery of mononuclear cells derived from bone marrow or peripheral blood has raised many promises for these patients to spare amputation[7]. The success of the first clinical trial based on transplantation of bone-marrow mononuclear cells suggested that cell-therapy is a feasible, safe and effective intervention for no-options patients[8,9]. However, in-


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tensive investigations are required to improve the efficacy of cell-based therapy[10].

There are two main challenges involved to improve the efficacy of cell-based therapy. The first is concerned with the poor graft survival due to the diseased environment and limited survival factors such as delivery of oxygen and nutrients[11]. Hence, the method which could elongate the retention time is supposed to greatly improve the efficiency of cell therapy. The second is the hyperbaric oxygen treatment (HBOT), which can increase oxygen tension in the ischemic area by 10-20 folds[12] and decrease oxygen diffusion, leading to enhancement of microenvironment. Furthermore, numerous studies[13] have reported the benefit of HBOT on angiogenesis by increasing vascular endothelial growth factor (VEGF) production, inhibition of inflammatory response in endothelial progenitor cells (EPCs)[14], enhancing wound healing[15] and improving the efficiency of cell therapy[16]. However, Li et al[17]suggest that HBOT only improved diabetic foot in short terms based on some under validated results.

Another challenging problem is the maturation of new vessels[6]. It was found[18] that vessels formed by cell transplantation are not so robust to meet the demand for oxygen and nutrients for the ischemia area. In such cases, combinations of cell therapy with gene therapy or protein therapy can promote cytoprotective or proangiogenic properties. It should be mentioned[19,20] that combination therapy has shown great success in generating stable and functional vessels.

Apelin-13 is one of the two most well-known growth factors associated with vessel maturation that could regulate the diameter of the vessel during angiogenesis and suppress hyperpermeability by promoting the proliferation and assembly of EPCs[21-23]. Besides cell therapy, gene delivery of apelin-13 has been reported[21] to improve vessels maturation.

We explored the efficacy of triple combined therapy: cell therapy, HBOT and apelin-13 gene delivery. Hyperbaric oxygen improved the microenvironment and apelin-13 enhanced vessel maturation together and such therapy promoted neovascularization of ischemic limbs in rats.

## Materials and Methods

### Materials

All animal procedures conformed with the National Institutes of Health (NIH), Guide for the Care and Use of Laboratory Animals. The study was further approved by the Animal Care and Committee of Ningxia Medical University.

### Animal Model for Lower-Limb Ischemia

Male Wistar rats (500±10 g) were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, Hubei, China). After 10% chloral hydrate (Shifeng Biological Technology, Shanghai, China; 300 mg/kg) was used for abdominal anesthesia, the incision was made along the ventral side of the left hind limb under aseptic conditions. The femoral arteries were ligated, whereas the groin together with the superficial femoral artery and femoral depth artery and its main branches were removed. The operation was ceased after hemostasis and suturing of the wound. The animals were then placed in the specific pathogen free (SPF)-level breeding room to observe the blood supply of the affected limb.

### Isolation and Identification of EPCs

Rats were stimulated with rat granulocyte colony stimulating factor (rG-CSF; PeproTech, Rocky Hill, NJ, USA) to mobilize EPCs from the bone marrow[24,25]; then, the whole bone marrow was isolated, cultured and identified as previously described[26,27]. Briefly, allogenic bone marrow mononuclear cells (BM-MNCs) were isolated by density gradient centrifugation (Cence, Changsha, China), suspended at a density of $2 \times 10^6$ cells/ml in the Endothelial Cell Growth Medium-2 Bullet Kit (Shanghai Biotechnology, Shanghai, China) and incubated in fibronectin-coated well plates at 37°C in an incubator (Heal Force, Shanghai, China) with 5% $CO_2$. After 7 days of incubation, the adherent BM-MNCs was treated with Dil-labeled acetylated low-density lipoprotein (DIL-acLDL, On Hailu Wen Biological Technology, Shanghai, China) and FITC labeled Ulex Europaeus Agglutinin I (FITC-UEA-1, On Hailu Wen Biological Technology, Shanghai, China), and observed under laser scanning confocal microscope (Carl Zeiss, Heidenheim, Germany).

### Flow Cytometric Analysis

The EPC single cell suspension was generated into $1\times10^6$ cells/ml. Then, the cell suspensions were incubated with anti-rat CD34-FITC (Haoran Biological Technology, Shanghai, China) and anti-CD133-APC (Haoran Biological Technology, Shanghai, China), respectively. 100 µl of cell suspension was incubated with 5 µl of antibody (Haoran Biological Technology, Shanghai,

China) in the dark. Then, the cells were washed and re-suspended by Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA). Lastly, the cells were analyzed with a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) to quantify the ratio of EPCs.

### Treatment and Transplantation

The studied rats were divided into five groups, with ten in each group: 1) negative control: injected with cell culture media, 2) BM-MNCs group: the cells were transferred without any extra treatment. 3) Apelin-13 group: BM-MNCs were incubated with apelin-13 (Phoenix Biotech, Beijing, China; 5 μmol/L) for 24 h before transplantation. 4) HBOT group: the rats were exposed to hyperbaric oxygen (Hongyuan Oxygen Industrial, Yantai, China) for 90 minutes per day for 7 consecutive days, and received an identical cure after the transplantation process. 5) Apelin-13 with HBOT group.

Ten days after operative excision of the artery, all the rats except those in the negative control group received a muscle injection at the ischemic limbs of $8 \times 10^6$ culture-expanded EPCs at 8 sites. The rats in the negative control group were identically injected with culture media used for EPC expansion.

### Hematoxylin and Eosin (HE) Staining

After 28 days of cell injection, the gastrocnemius muscles of the ischemic limb were harvested for subsequent analysis. The muscles were further sliced into small sections of 5 μm thickness following paraffin embedding. After completing both conventional dewaxing and gradient ethanol hydration, the prepared sections were stained with hematoxylin (Solarbio, Beijing, China) for 2 min subsequently with eosin (Solarbio, Beijing, China) for 1 min, and dehydrated by gradient ethanol. The sections were sealed using neutral gum (Solarbio, Beijing, China) after being cleaned in xylene and then observed under a light microscope (Olympus, Tokyo, Japan).

### Immunohistochemistry

The cluster of differentiation (CD)31, CD34 and vascular endothelial growth factor receptor 2 (VEGFR-2) were selected as the representative endothelial cells (ECs) markers[28]. The sections were obtained by the abovementioned method and treated with $H_2O_2$. The antigen was retrieved by high pressure with the addition of sodium citrate solution. The sections were blocked with goat serum and incubated with the primary anti-rat-CD31/CD34/VEFGR-2 monoclonal antibodies (Abcam, Cambridge, MA, USA; diluted 1: 200) and horseradish peroxidase (HRP) goat anti-rat Ig as the secondary antibody (Abcam, Cambridge, MA, USA). Labeling was performed by incubating the slides with 3,3'-diaminobenzidine (DABI; ASPEN, Wuhan, China) and stained with hematoxylin. Finally, the sections were observed under a fluorescence microscope (Carl Zeiss, Heidenheim, Germany) after sealing with neutral gum (Solarbio, Beijing, China).

### Real Time Quantitative-Polymerase Chain Reaction (RT-qPCR) Analysis

Total RNA was isolated from the tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentrations were measured by a spectrophotometer (Invitrogen, Carlsbad, CA, USA). The isolated samples were characterized by an A260/A280 ratio >1.90. Complementary DNA (cDNA) was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Otsu, Shiga, Japan). Real Time-quantitative Polymerase Chain Reaction (RT-PCR) was performed on Life Technologies' StepOneTM Real Time-PCR instrument, and each sample was made up of 3 replicates using the SYBR® Premix Ex TaqTM kit (TaKaRa, Otsu, Shiga, Japan) in the presence of the appropriate primers (GeneCreate, Wuhan, China; Table I). The following Real Time-qPCR conditions were used for amplification by a Real Time-qPCR detecting

**Table I.** The primer sequence of GAPDH, CD31, CD34 and VEGFR-2.

| Primer Name | | Primer Sequence |
|---|---|---|
| GAPDH | Forward | 5`-CGCTAACATCAAATGGGGTG-3` |
| | Reverse | 5`-TTGCTGACAATCTTGAGGGAG-3` |
| CD31 | Forward | 5`-GATCTCCATCCTGTCGGGTAAC-3` |
| | Reverse | 5`-GTGTCATTCACGGTTTCTTCGT-3` |
| CD34 | Forward | 5`-CCACAGACTTACCCAACCGTC-3` |
| | Reverse | 5`-CCTCGGATTCCTGAACATTTG-3` |
| VEGFR-2 | Forward | 5`-TTCATAATAGAAGGCGTCCAGG-3` |
| | Reverse | 5`-GCATCATAAGGCAAGCGTTC-3` |

system (Bio-Rad, Hercules, CA, USA): denaturation at 95°C for 1 min; 40 cycles at 95°C for 15 sec, 58°C for 20 sec, and 72°C for 45 sec; 1°C increased per 20 s in the melting curve from 60°Cto 95°C. All transcripts were analyzed in parallel on at least three separate occasions in a thermal cycler. Data were evaluated using the comparative CT (ΔΔCT) method. The primer sets were presented in Table I.

### Western Blot Analysis

The tissue samples were rinsed 3 times with pre-cooled PBS buffer (HyClone, South Logan, UT, USA) to remove blood stains and cut them into small pieces for placement in a homogenizer. Protein extraction reagent (Boster Biological Technology, Wuhan, China) was added to dissolve the tissue and grind them in an ice bath. The homogenate was transferred to a centrifuge tube, fully vortexed and the tube was placed in the ice bath for 30 min to ensure the complete lysis of the homogenate. Centrifugation at 12000 rpm for 5 min at 4°C (Cence, Changsha, China) followed to collect the supernatant to make the total protein solution. The micro-bicinchoninic acid (BCA) protein assay (ASPEN, Wuhan, China) was used to determine the sample concentration to ensure that the total protein loading per sample was 40 μg. An appropriate amount of 5× protein loading buffer (ASPEN, Wuhan, China) was added to the protein sample and boiled at 100°C for 5 min. The protein lysates were separated by electrophoresis (Bio-Rad, Hercules, CA, USA) in 8-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (ASPEN, Wuhan, China), transferred onto a polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membrane, and blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-Buffered Saline and Tween (TBST; Sigma-Aldrich, St. Louis, MO, USA; 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 5% TBS). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Abcam, Cambridge, MA, USA) was used as an internal control. The PVDF membranes were then probed with specific primary antibodies (Abcam, Cambridge, MA, USA) overnight at 4°C, washed with TBST three times (10 min/wash), and blocked for 2 h at room temperature with the appropriate secondary antibody (Abcam, Cambridge, MA, USA). The membranes were imaged, and the density of each band was analyzed using GelDoc software (Bio-Rad, Hercules, CA, USA).

### Statistical Analysis

The results were expressed as means ± SDs, and 95% confidence intervals (CI) were calculated, if necessary. Comparisons of the angiogenic factors among treatment groups, between control and each treatment, were tested using the mixed model. Due to repeated measurements per subject *p*-values were adjusted with Bonferroni's correction. All statistical analysis was performed with SAS version 9.13, and a $p$-value$<0.05$ was considered statistically significant.

## Results

### Identification and Quantification of EPCs

After seven days of cultivation, the isolated BM-MNCs were stained by DIL-acLDL and FITC-UEA-1 and observed under a fluorescence microscope. Cells taking DIL-acLDL were emitting red while green for those taking FITC-UEA-1 as presented in Figure 1A. Differentiating EPCs could endocytose acLDL and bind UEA-1. In the merged figure, the yellow cells are confirmed as the EPCs. Flow cytometry map indicates the ratio of EPCs in the isolated BM-MNCs and the 14-days-cultured BM-MNCs (Figure 1B).

As revealed from Fluorescent-activated cell sorter (FACS), 83.8% and 21.7% of BM-MNCs were antigenically defined cells for the treated and controlled group, respectively. The CD34 marker of ECs was expressed on 74.39% of the cultured mononuclear cells while 0.85% for the control group. The CD133 marker of EPCs was 77.7% for the treatment group and 21.2% for the control group. The double-positive CD34 and CD133 was 0.32% and 68.3% for the control and treated group, respectively. EPCs took more than 80% in the antigenically defined BM-MNCs, which was qualified for transplantation[27].

### Triple Therapeutic Strategy Enhances the Development of New Vessels in Ischemic Limbs.

To directly assess the effects on vascularization by HE staining, we evaluated different therapeutic effects by the density of newly formed vessels in the ischemic area and averaged the density by three different areas. All vessels are marked by black arrows, and the red area is muscle (Figure 2A). The average vessel numbers for the five groups are as follows: 1.33 for the negative control; 2.33 for the BM-MNC

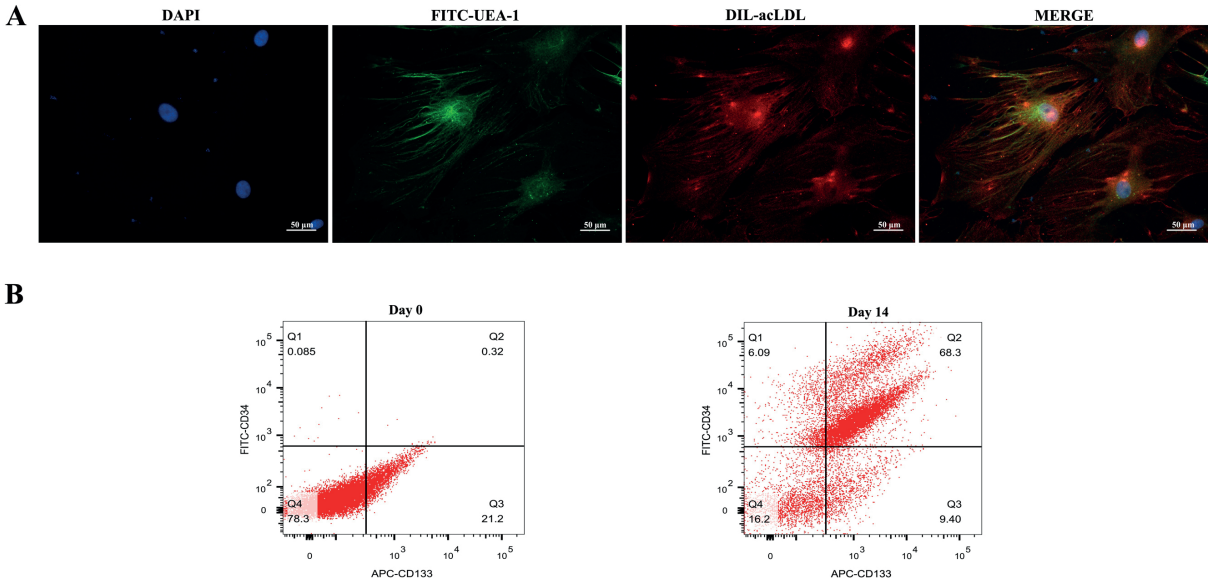


**Figure 1.** Identification and quantification of EPCs. ***A,*** Identification of cultured BM-MNC as differentiating EPCs. Blue for nuclear staining by DAPI. Red for DIL-acLDL and green for FITC-UEA-1. The cells showing red and green merged signals were differentiated EPCs. Scale bars: 50μm. ***B,*** Flow cytometry map indicates the ratio of EPCs in the isolated BM-MNCs and the 14 days cultured BM-MNCs.

transplantation group; 4.66 for the apelin-13 group with BM-MNCs transplantation; 2.66 for the hyperbaric group with BM-MNCs transplantation; 8.00 for the triple therapy groups (Figure 2B).

Notably, HE staining revealed a profoundly increased vessels density in the triple therapy groups, and the vessels number was almost 3 times as much as that in the BM-MNCs transplantation group and was over 6 times more than that in the control group. While apelin-13 had limited effect to promote vessel formation and hyperbaric oxygen made little difference compared with the BM-MNCs group, suggesting the combination effect is better than the improvement made by the two single elements. Vessel density in all treated groups had different levels of improvement than the negative control, indicating the cell therapy was successful in our experiment.

### *Combinatorial Treatment of Apelin-13 and Hyperbaric Oxygen Promotes Differentiation of EPCs*

To directly assess the effects of different treatments on EPCs, we examined the expression levels of CD31 (Figure 3A), CD34 (Figure 3B) and VEGFR-2 (Figure 3C) in muscle sections by immunohistochemistry. As shown in Figure 3A, the first row was nucleus stained by 4',6-diamidino-2-phenylindole (DAPI), the second row was positive area for CD31, the merged blue and

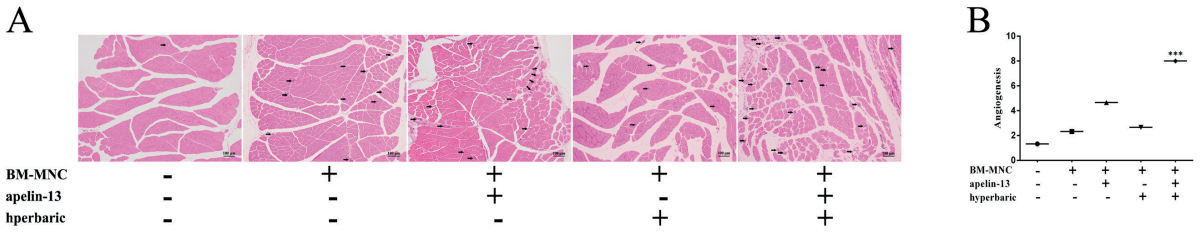


**Figure 2.** HE staining for examination of neovascularization *in vivo*. ***A,*** The muscle is red, and blood vessels are bluish violet. Scale bars: 100 μm. ***B,*** Statistics of vessels number in one view. The number was averaged by 8 different views. (***$p < 0.001$ compared with the BM-MNCs transplantation group).

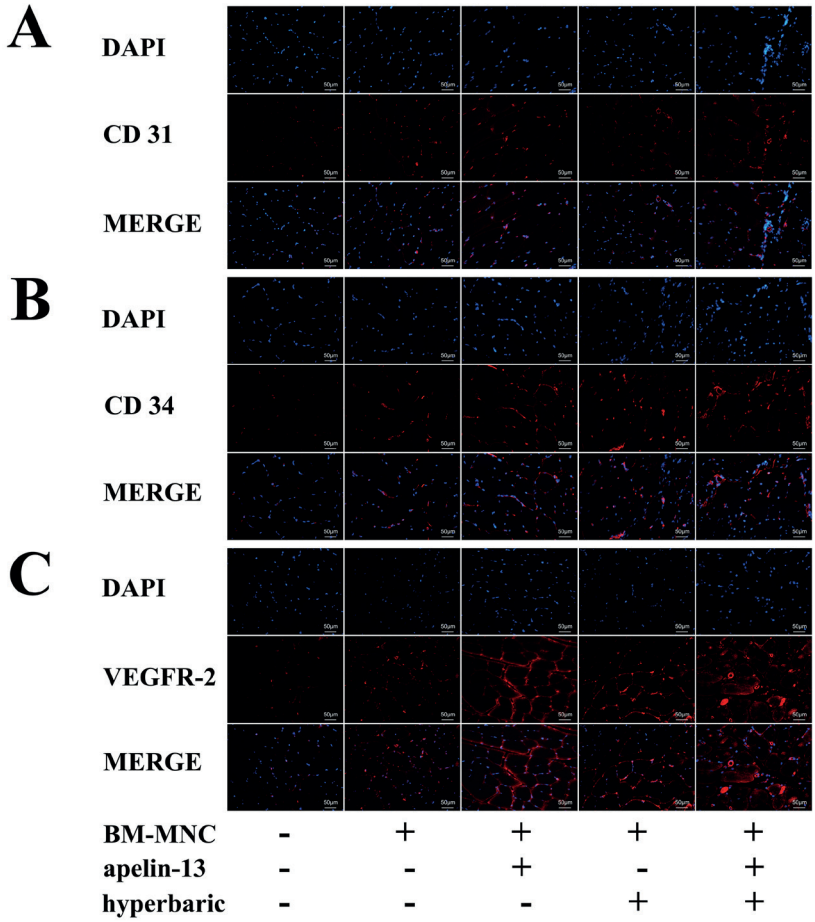


**Figure 3.** Immunohistochemistry examination of the expression of ECs markers *in vivo*. ***A-C***, indicates the immunohistochemistry for CD31, CD34, and VEGFR-2 respectively. The nuclear was stained blue by DAPI. The CD31, CD34, and VEGFR-2 were marked red. The cells with blue nuclear and strong red membrane were regarded as ECs. Scale bars: 50μm.

strong red signals indicated positive ECs. The expression level of CD31 in the triple therapy was dominant among all groups, followed by apelin-13, while a few positive CD31 expressions were observed in control, the BM-MNCs and hyperbaric group. As for the positive expression of CD34, the trend was almost the same with that for CD31. The overview expression of VEGFR-2 was stronger than CD31 and CD34. With no exception, the expression of the triple group had a profoundly increasing positive area compared to other groups, and both the apelin-13 and the hyperbaric groups had more positive area than the BM-MNCs group. Compared to the little effects on CD31 and CD34, hyperbaric oxygen promoted more VEGFR-2 expression, which has been reported in a previous study[13]. Data from the positive expression area of the three markers were consistent with the density of the vessels number, with the most significant improvement of ischemia from the triple therapy, followed by the apelin-13 group, while very limited effects caused by HBOT with BM-MNCs transplantation.

### *Combinatorial Treatment Results in High Expression of ECs Markers in Both Gene and Protein Level*

To predict the possible formation of new vessels, we analyzed the gene and protein expressions of three ECs markers CD31, CD34 and VEGFR-2. First, Real Time-qPCR was conducted to quantify messenger ribonucleic acid (mRNA) expressions and repeated 3 times for each sample. The expression of CD31, CD34, and VEGFR-2 mRNA levels are tabulated in Table II and represented in Figure 4A.

Compared with the negative control, the combinatorial treatment group showed a significant increase in the expression of the targeted markers CD31 ($p < 0.001$), CD34 ($p < 0.001$) and VEG-

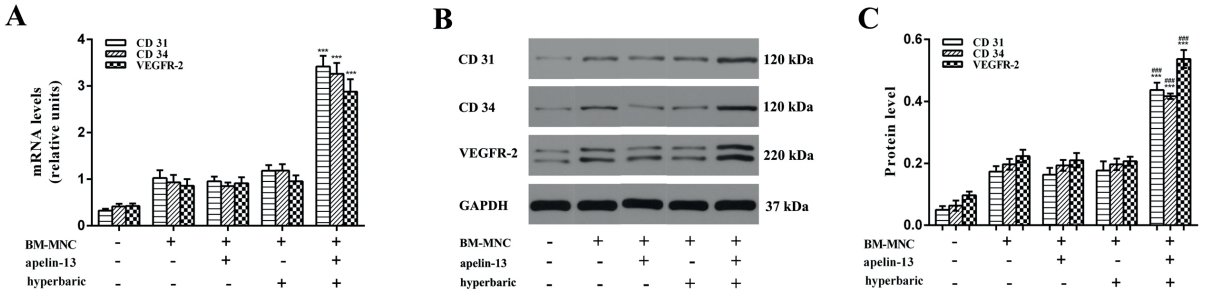


**Figure 4.** The expressions of CD31, CD34, and VEGFR-2 on mRNA and protein levels. ***A,*** The mRNA level of CD31, CD34, VEGFR-2 in different groups. In the three paralleling panels, the left one is CD31, middle for CD34 and right for VEGFR-2. ***B,*** The protein level of CD31, CD34, VEGFR-2 in different groups. ***C,*** The gray value of CD31, CD34, and VEGFR-2 protein bands in the above Western blot. ($^{***}p < 0.005$ compared with the other 4 groups. $###p < 0.001$ compared with the BM-MNCs transplantation group).

FR-2 ($p < 0.001$), indicating the superior potential of neovascularization in the combination group. For single treatment, apelin-13 or hyperbaric oxygen with cell transplantation, there was little difference between the two groups with the BM-MNCs group, suggesting low efficacy of the single treatment on cell-based therapy.

Assessment of the protein expression was investigated by Western blot and repeated 3 times for each animal sample (Figure 4B). The protein levels of CD31, CD34, and VEGFR-2 among the five groups were presented in Table III and Figure 4C.

The protein trend of comparison in the five groups was similar to mRNA expression levels (Figure 4 A). The expressions of the three markers were almost the same in the BM-MNCs, apelin-13 and hyperbaric groups, while a significant increase ($p < 0.001$) of CD31, CD34, and VEGFR-2 was observed in the triple therapeutic compared with the negative group.

## Discussion

CLLI patients remain miserable to be treated successfully, especially when they are accompanied by some comorbidities. Gene therapy and cell-based therapy have been suggested as an effective and safe treatment for CLLI; however, more enhancements are required due to the complexity of vascular formation and individual situation[1,6]. The combinatorial cell therapy is a promising therapeutic strategy and various strategies have been used to enhance therapeutic efficacy involving protein injection or gene co-delivery such as angiogenesis factors ang-1[29] and pulsed focused ultrasound with cells[30]. Therefore, in this study, we explored an effective and practical combination of cell-based therapy together with apelin-13 and HBOT. Apelin is an endogenous ligand of an orphan G protein-coupled receptor putative receptor protein related to the angiotensin receptor

**Table II.** The mRNA levels of CD31, CD34 and VEGFR-2 among the five groups.

| Marker | Negative control | BM-MNCs | Apelin-13 | Hyperbaric oxygen | Apelin-13 + hyperbaric oxygen |
|---|---|---|---|---|---|
| CD31 | 0.32±0.07 | 1.03±0.28 | 0.96±0.17 | 1.18±0.21 | 3.42+0.40 |
| CD34 | 0.42+0.10 | 0.93±0.28 | 0.85±0.13 | 1.18±0.24 | 3.26+0.41 |
| VEGFR-2 | 0.43±0.09 | 0.86±0.25 | 0.91±0.23 | 0.96±0.21 | 2.88±0.46 |

**Table III.** The protein levels of CD31, CD34 and VEGFR-2 among the five groups.

| Marker | Negative control | BM-MNCs | Apelin-13 | Hyperbaric oxygen | Apelin-13 + hyperbaric oxygen |
|---|---|---|---|---|---|
| CD31 | 0.05±0.02 | 0.17±0.03 | 0.16±0.04 | 0.18±0.05 | 0.44±0.04 |
| CD34 | 0.07+0.03 | 0.20±0.03 | 0.19±0.03 | 0.20±0.03 | 0.42+0.02 |
| VEGFR-2 | 0.10±0.02 | 0.22±0.04 | 0.21±0.04 | 0.21±0.03 | 0.54±0.05 |

AT1 (APJ) and apelin-13 is known to be vital in vascular diseases[31]; hyperbaric oxygen has been proposed to improve the chance of healing in the clinical treatment of diabetes[17]. According to our results, the triple combination showed remarkable progress in treatment. The major findings of this study can be summarized as follows: 1) Compared with cell-based therapy, the combination with apelin-13 or hyperbaric oxygen alone have shown a little improvement of new vessels formation, which is consistent with previous reports concerning the benefits of the two elements on neovascularization. 2) The two elements have promoted the surface expression of different EPCs markers, while little difference is made on the expression of mRNA levels. 3) Compared with the negative and cell therapy groups, the triple therapeutic group combined with both apelin-13 and hyperbaric oxygen showed remarkable effects on promoting the formation of new vessels, as well as the expression of EPC markers in three aspects. Though limited data showed the positive effects of the triple therapy, and we neglect the long-term effects of treatment and the deeper mechanism; there is no doubt about the notable improvement achieved by the triple treatment. About the efficacy of triple therapy, which is more than superimposed effects of apelin-13 and HBOT, we suggest that the two elements play different and complementary roles during the process of vessel formation, thus making the process more smoothly and achieving an unexpected success.

Neovascularization is a rather complex process including vasculogenesis and angiogenesis, and the process requires many regulatory factors with the microenvironment. Vasculogenesis occurs when EPCs migrate to ischemia tissue and differentiate to form new vessels while angiogenesis is the sprouting to connect with existed blood supply and stabilization of new vessels. HBOT mainly improves the worse ischemia microenvironment by raising oxygen intension, thus making a good environment for graft cells. Additionally, hyperbaric oxygen could upregulate the active production of endothelial nitric oxide synthase (eNOS), inducing vascular remodeling, angiogenesis and EPCs migration and proliferation, as well as VEGF, which is the most influential growth factor in angiogenesis[32-34].

Apelin-13 plays a vital role in all steps of angiogenesis. Here we only use apelin-13 for the treatment of BM-MNCs, so the treatment has nothing to do with angiogenesis after transplantation. However, during the *in vitro* expansion process, the level of phosphorylated platelet-derived growth factor receptors (PDGFR)-β, which recruits mural cell and promotes vessel maturation[35,36], is decreased in the EPCs pathway, as well as impairing their angiogenic potential by inhibiting phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling[37], which is essential for VEGF-mediated postnatal angiogenesis[38]. Apelin-13 is reported to induce the upregulation of PDGFR-β[39] and Akt, as the upstream regulator[31]. Meanwhile, apelin-13 could increase phorypolation of Akt and eNOS and upregulate VEGF expressions[40]. To our best knowledge, hyperbaric oxygen and apelin-13 promote neovascularization from different aspects, but there is no report about their synergistic effect.

Recently, the combinatorial treatment of apelin-13 with hypoxia-preconditioning peripheral blood mononuclear cells (PB-MNCs) was stated as an effective therapeutic strategy in peripheral ischemia[39]. Both hypoxia and hyperbaric conditions have been reported to upregulate VEGF and wound healing *in vitro* studies[33]; however, hypoxia could only stimulate angiogenesis without sustaining the process[41]. Considering the long-term effects, hyperbaric is preferable than hypoxia since angiogenesis can successfully proceed only when enough oxygen and VEGF released at high oxygen tension[42]. Meanwhile, we used apelin-13 to treat the BM-MNCs instead of infusion in animals, making the treatment more practical to be applied in clinical settings for no need to consider the adverse effects of apelin-13 on patients.

## Conclusions

We demonstrated, for the first time, that the combination of cell-based therapy with apelin-13 and hyperbaric oxygen could efficiently promote neovascularization in ischemic limb. Besides the two treatments, BM-MNCs with apelin-13 and the animal or human with hyperbaric oxygen, are considered practical in both experimental and clinical trials, thus making the therapy a promising therapeutic option for some CLLI patients. However, further clarification is needed to explore the mechanism of the triple therapy involved in the neovascularization.

### Conflict of Interest

The Authors declare that they have no conflict of interest.

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