Original Article

Hyperbaric oxygen facilitates the effect of endothelial progenitor cell therapy on improving outcome of rat critical limb ischemia


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Abstract: We tested the hypothesis that hyperbaric oxygen (HBO) (100% oxygen/2.4 atmospheres) facilitated the effect of autologous endothelial progenitor cell (EPC) therapy on restoring the blood flow in rat critical-limb ischemia (CLI). Adult-male-SD rats (n = 8/group) were categorized into group 1 [sham control (SC)], group 2 (CLI-treated with culture medium), group 3 [CLI-intermittent HBO (3 h/day for 5 consecutive days after CLI)], group 4 (CLI-EPC/2.0 × 10^6 cells), and group 5 (CLI-HBO-EPC). By day 5 after CLI, flow cytometry showed that the circulating EPC (Sca-1/CD31+/C-kit/CD31+/CD34+) levels were highest in group 5 and lowest in group 2 (all P < 0.001). By day 14, laser Doppler demonstrated that the ratio of blood flow (i.e., CLI to normal hind-limb) was highest in group 1, lowest in group 2 and significantly higher in group 5 than in groups 3 and 4 (all P < 0.0001). The protein expressions of endothelial-cell biomarkers (CD31/vWF/eNOS), and numbers of endothelial-cell markers (CD31+/vWF+) and small vessels exhibited a similar pattern to blood-flow ratio among five groups, whereas the angiogenesis parameters in protein (CXCR4/SDF-1α/HIF-1α/VEGF) and cellular (HIF-1α/SDF-1α/CXCR4+) levels were progressively increased from groups 1 to 5 (all P < 0.0001). The protein expression of apoptotic (mitochondrial-Bax/cleaved-caspase-3/PARP), fibrotic (p-Smad3/TGF-ß) and mitochondrial-damaged (cytosolic-cytochrome C) exhibited an opposite pattern, whereas the protein expressions of anti-fibrotic (BMP-2/p-Smad1/5) and mitochondrial integrity (mitochondrial-cytochrome C) exhibited an identical pattern of ratio of blood flow among the five groups (all P < 0.0001). Combined HBO-EPC therapy is superior to either one alone in improving ischemia in rodent CLI.

Keywords: Critical limb ischemia, hyperbaric oxygen, endothelial progenitor cells, angiogenesis, apoptosis, fibrosis

Introduction

Aging-related chronic diseases, such as cardiovascular disease and peripheral arterial occlusive disease (PAOD), not only incur huge public healthcare costs but also cause unacceptably high morbidity and mortality. Atherosclerotic PAOD, one of the major manifestations of systemic atherosclerosis [1] such as myocardial infarction and ischemic stroke, has been identified to affect 12% of the adult population and up to 20% of the elderly [2]. Patients with PAOD may develop critical limb ischemia (CLI) at the late stage of the disease [2, 3]. CLI commonly occurs when arterial blood flow is restricted so severely that perfusion of capillary beds is inadequate to sustain tissue viability [4, 5]. Importantly, research has established that many
patients are asymptomatic prior to the development of CLI [6, 7] which is an obstacle to early diagnosis and early treatment for the purposes of slowing or abolishing disease progression and development of complications.

The compensatory mechanisms of PAOD involve generation of angiogenesis factors such as vascular endothelial growth factor, nitric oxide, hypoxic inducible factor (HIF)-1α, and capillary sprouting as well as arteriogenesis [8] for alleviation of the blood flow deprivation. However, in patients with CLI, these mechanisms have been exhausted and/or are severely defective. Treatment for CLI is still a formidable challenge to clinicians [9]. Without appropriate treatment, the one-year mortality rate for CLI is as high as 25% [10]. Failure in salvaging this disease can lead to limb loss and high cost of patient care following amputation. Despite surgical or endovascular revascularization being utilized for the treatment of CLI with an acceptable success rate, for those patients who are not candidates for surgical or endovascular intervention and those with failure of revascularization or bypass occlusion, clinical outcomes remain dismal. Therefore, an alternative strategy for the treatment of CLI patients who are refractory to conventional therapy is necessary.

Hyperbaric oxygen (HBO) therapy is a traditional therapy for patients with ischemic PAOD [11]. The underlying mechanism of HBO therapy involved in improving ischemic PAOD has been proposed to be mainly through an increase of vascular wall permeability and productions of HIF-1α and stromal cell-derived factor (SDF)-1α that enhance the angiogenesis and blood flow in the ischemic area [12]. Unsatisfactorily, overall limb salvage and progression of the ischemic process was not significantly decreased in patients receiving HBO therapy [13]. This may be because of diffusion of oxygen into the ischemic organ creating a hyperbaric environment is extremely limited [14, 15]. Therefore, increasing the angiogenesis or neovascularization and repair of endothelial function in the ischemic area may play a principal role in increasing oxygen diffusion into ischemia that contributes to likelihood of successfully salvaging the limbs of CLI patients.

Endothelial progenitor cells (EPCs) have been extensively investigated and are known to have the capacity for angiogenesis/vasculogenesis, and the growth of new vessels from bone marrow-derived progenitor cells [16]. It is also known that EPCs play a pivotal role in wound healing [17]. Additionally, EPCs have been shown to actively home to wounds and ischemic areas for establishment of neovascularization and restoration of blood flow [17-22]. During the healing process, EPC trafficking/homing is directed by hypoxic tissue gradients via HIF-1α signals that enhance SDF-1 expression [23]. However, the intrinsic response to pathophysiologic triggers for EPC homing to the ischemic area is often insufficient due to severe ischemia and an extremely hypoxic environment [17]. Accordingly, a combination of EPC and HBO therapy could be an alternative option for salvaging the CLI patients.

Materials and methods

Ethics and animal studies

All animal experimental protocols and procedures were approved by our institutional Animal Care and Use Committee at Kaohsiung Chang Gung Memorial Hospital (Affidavit of Approval of Animal Use Protocol No. 2016011501) and executed in accordance with the Guide for the Care and Use of Laboratory Animals [The Eighth Edition of the Guide for the Care and Use of Laboratory Animals (NRC 2011)].

Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal facility in our hospital with controlled temperature and light cycles (24°C and 12/12 light cycles).

Animal model of critical limb ischemia, animal grouping, and treatments

The procedure and protocol were based on our previous reports [24, 25]. In brief, pathogen-free, adult male Sprague Dawley (SD) rats weighing 325-350 g (Charles River Technology, BioLASCO, Taiwan) in CLI groups were anesthetized by inhalation of 2.0% isoflurane. The rats were placed in a supine position on a warming pad at 37°C with the left hind limbs shaved. Under sterile conditions, the left femoral artery, small arterioles, circumferential femoral artery and veins were exposed and ligated over their proximal and distal portions before removal. To
avoid the presence of collateral circulation, the branches were removed together. For laser Doppler study, 8 rats in each group were utilized and 6 rats in each group were used for cellular-molecular assessment. For animals that served as normal controls, the arteries were only isolated without ligation.

Forty SD rats were categorized into five groups: sham-operated control (SC), CLI (only treated with culture medium 1.0 cc over the femoral area), CLI+HBO [i.e., intermittent HBO therapy/3 h per day for 5 consecutive days after the CLI procedure), CLI+EPC [2.0 × 10^6 cells, i.e., 1.2 × 10^6 from penis vein injection and 0.8 × 10^6 by four sites of intramuscular injections in CLI rea 3 h after CLI procedure]), and CLI+HBO+EPC. The administration of EPCs to the animals by two different routes was based on our recent report [26]. This modality was more reliable for the EPC homing to the CLI area.

**Measurement of blood flow with laser Doppler**

The procedure and protocol were based on our previous reports [24, 25]. In brief, rats were anesthetized by inhalation of isoflurane (2.0%) prior to CLI induction and at days 2, 7, 14 after CLI induction prior to sacrifice. The rats were placed supine on a warming pad (37°C) and blood flow was detected in both inguinal areas by a laser Doppler scanner (moorLDLS, Moor Instruments, UK). The ratio of flow in the left (ischemic) leg and right (normal) leg was computed. By day 14, the rats were euthanized and the quadriceps muscle was collected for individual study.

**Hyperbaric oxygen therapy**

The procedure and protocol of hyperbaric oxygen (HBO) therapy was based on a previous report with minimal modification [27]. Briefly, to induce tissue-level hyperoxia, SD rats were subjected to HBO administration in an animal tabletop chamber (Piersol-Dive, model 4934) with the animals exposed to 100% oxygen at 2.4 atmospheres absolute (ATA) for 90 minutes (3 h/day for 5 consecutive days).

**Peripheral blood was collected and cultured for autologous endothelial progenitor cells**

The procedure and protocol for EPC isolation and culture were based on our previous reports [24, 28] with some modification. In brief, animals in groups 4 and 5 were anesthetized with inhalational 2.0% isoflurane at day 21 prior to CLI induction to collect peripheral blood for culturing EPCs and autologous transfusion. Isolated mononuclear cells from peripheral blood were cultured in a 100-mm diameter dish with 10 mL DMEM culture medium containing 10% FBS for 21 days. Flow cytometric analysis was performed for identification of cellular characteristics (i.e., EPC surface markers) after cell-labeling with appropriate antibodies on day 21 of cell cultivation prior to implantation.

**Flow cytometric quantification of endothelial progenitor cells**

Tail vein route was adopted for blood sampling at baseline and days 5 and 14 after CLI procedure. After treatment with red blood cell-lysing buffer, the cells remained were labeled with appropriate antibodies. Flow cytometric analysis for identification of cell surface markers was performed based on our recent reports [28, 29]. Briefly, the cells were immunostained for 30 minutes with monoclonal antibodies against primary antibodies, including CD 31-PE, CD 31-Alexa Fluor® 488 (BD Biosciences), c-kit (BD Biosciences), Sca-1-PE (R&D), VE Cadherin (Abcam) and CD34-PE (BD Biosciences). Secondary detection was performed using appropriate IgG-FITC (BD Biosciences). Isotype-identical antibodies (IgG) served as controls. Flow cytometric analyses were performed by utilizing a fluorescence-activated cell sorter (Beckman Coulter FC500 flow cytometer).

**Preparation of a nitrite standard reference curve**

First, 1 ml of a 100 μM nitrite solution was prepared by diluting the provided 0.1 M nitrite standard 1:1,000 in buffer used for the experimental samples, followed by dispensing 50 μl of the appropriate matrix or buffer into the wells in rows B-H. Then, 100 μl of the 100 μM nitrite solution was added to the remaining 3 wells in row A. We immediately performed 6 serial two-fold dilutions (50 μl/well) in triplicate down the plate to generate the nitrite standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 μM), discarding 50 μl from the 1.56 μM set of wells. The final volume in each well was 50 μl with the nitrite concentration in the range 0-100 μM.
Measurement of nitric oxide by Griess assay

First, the sulfanilamide solution and NED Solution were allowed to equilibrate to room temperature (15-30 minutes). Then, 50 μl of each experimental sample was added to wells in duplicate or triplicate. Using a multichannel pipettor, 50 μl of the sulfanilamide solution was dispensed to all experimental samples and wells containing the dilution series for the nitrite standard reference curve, followed by incubation for 5-10 minutes at room temperature and protected from light. Using a multichannel pipettor, 50 μl of the NED Solution was dispensed to all wells, followed by incubation at room temperature for 5-10 minutes with protection from light. A purple/magenta color was identified immediately. Finally, absorbance was measured within 30 minutes in a plate reader with a filter between 540 nm and 690 nm.

Autologous EPC implantation

EPCs (2.0 × 10⁶ cells) were labeled by CM-Dil (Vybrant Dil cell-labeling solution, Molecular Probes) 30 minutes before CLI induction and then injected intra-muscularly into the hind limb ischemic area (0.8 × 10⁶ cells) and intra-venously administered (1.2 × 10⁶ cells from the penis vein) 3 h after CLI procedure, once, before animal recovery from anesthesia.

Immunofluorescence staining

IF staining was performed for the examinations of CD31+, von Willebrand factor (vWF)+, CXCR4+, and SDF-1α+ cells (n = 6 for each group) using respective primary antibodies based on our recent study [24, 28, 29]. Irrelevant antibodies were used as controls in the current study.

Western blot analysis

The procedure and protocol of western blot analysis was based on our previous reports [24, 25, 28]. In brief, equal amounts (30 mg) of protein extracts from ischemic quadriceps of the animals were loaded and separated by SDS-PAGE using 12% acrylamide gradients. The membranes were incubated with monoclonal antibodies against vascular cell adhesion molecule CXCR4 (1:1000, Abcam), VEGF (1:1000, Abcam), stromal cell-derived growth factor (SDF)-1α (1:1000, Cell Signaling), connexin 43 (Cx43) (1:2000, Chemicon), cytochrom c (Cyt c) (1:2000, BD), endothelial nitric oxide synthase (eNOS) (1:1000, Abcam), Bax (1:1000, Abcam), hypoxic inducible factor (HIF)-α (1:500, Abcam) and Bcl-2 (1:200, Abcam). Signals were detected with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG. Proteins were transferred to nitrocellulose membranes which were then incubated in the primary antibody solution (anti-DNP 1:150) for two hours, followed by incubation with second antibody solution (1:300) for one hour at room temperature. The washing procedure was repeated eight times within 40 minutes. Immunoactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) which was then exposed to Biomax L film (Kodak). For quantification, ECL signals were digitized using Labwork software (UVP).

Vessel density in limb ischemic area

Immunohistochemical (IHC) staining of blood vessels was performed (n = 6 for each group) with α-smooth muscle actin (SMA) (1:400) as primary antibody at room temperature for 1 h, followed by washing with PBS three times. Ten minutes after the addition of the anti-mouse-HRP conjugated secondary antibody, the tissue sections were washed with PBS three times. Then 3,3′diaminobenzidine (DAB) (0.7 gm/tablet) (Sigma) was added, followed by washing with PBS three times after one minute. Finally, hematoxylin was added as a counter-stain for nuclei, followed by washing twice with PBS after one minute. Three sections of quadriceps were analyzed in each rat. For quantification, three randomly selected HPFs (× 100) were analyzed in each section. The mean number per HPF for each animal was then determined by summation of all numbers divided by 9.

Statistical analysis

Quantitative data are expressed as means ± SD. Statistical analysis was adequately performed by ANOVA followed by Bonferroni multiple comparison post hoc test. Statistical analysis was performed using SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC). A probability value of less than 0.05 was considered statistically significant.
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Results

Time courses of circulating levels of endothelial progenitor cells (Figure 1)

At day 0, flow cytometric analysis demonstrated that the circulating levels of C-kit/CD31+, Sca-1/CD31+ and CD34+ cells, three surface markers of EPCs, did not differ among the groups 1 (SC), 2 (CLI), 3 (CLI+HBO), 4 (CLI+EPC) and 5 (CLI-HBO-EPC).

By day 5 after the CLI procedure, the circulating level of C-kit/CD31+ cells was highest in group

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Figure 1. Flow cytometric analysis for identifying the serial changes of circulating levels of endothelial progenitor cells. A. Analytical result of the number of C-kit/CD31+ cells at day 0, $P > 0.5$. B. Analytical result of number of Sca-1/CD31+ cells at day 0, $P > 0.5$. C. Analytical result of number of CD34+ cells at day 0, $P > 0.5$. D. Analytical result of number of C-kit/CD31+ cells at day 5, * vs. other groups with different symbols ($\dagger$, †, §, ¶), $P < 0.001$. E. Analytical result of number of Sca-1/CD31+ cells at day 5, * vs. other groups with different symbols ($\dagger$, †, §, ¶), $P < 0.0001$. F. Analytical result of number of CD34+ cells at day 5, * vs. other groups with different symbols ($\dagger$, †, §, ¶), $P < 0.0001$. G. Analytical result of number of C-kit/CD31+ cells at day 14, * vs. other groups with different symbols ($\dagger$, †, §, ¶), $P < 0.001$. H. Analytical result of number of Sca-1/CD31+ cells at day 14, * vs. other groups with different symbols ($\dagger$, †, §, ¶), $P < 0.001$. I. Analytical result of number of CD34+ cells at day 14, * vs. other groups with different symbols ($\dagger$, †, §, ¶), $P < 0.0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). SC = sham control; CLI = critical limb ischemia; HBO = hyperbaric oxygen; EPC = endothelial progenitor cell.
5 and lowest in group 2, significantly higher in group 4 than in groups 1 and 3 and significantly higher in group 1 than in group 3. Additionally, circulating level of Sca-1/CD31+ cells was highest in group 5, lowest in group 2, significantly higher in group 4 than in groups 1 and 3, and significantly higher in group 3 than in group 1. Furthermore, the circulating level of CD34+ cells was highest in group 5 and lowest in group 2, significantly higher in groups 1 and 4 than in group 3, but it exhibited no difference between groups 1 and 4.

By day 14 after CLI procedure, the circulating level of C-kit/CD31+ cells was highest in group 5 and lowest in group 2, significantly higher in groups 1 and 4 than in group 3, but it showed no difference between groups 1 and 4. Additionally, circulating level of Sca-1/CD31+ cells was highest in group 5, lowest in group 2, significantly higher in group 4 than in groups 1 and 3, and significantly higher in group 1 than in group 3. Furthermore, the circulating level of CD34+ cells was highest in group 5 and lowest in group 2, significantly higher in groups 1 and 4 than in group 3, but it exhibited no difference between groups 1 and 4.

Serial changes of ratio of ischemic/normal blood flow (INBF) measured by laser doppler scan (Figure 2)

By day 1 after CLI procedure, the ratio of INBF was significantly higher in group 1 than in groups 2 to 5, but showed no difference among these four groups.

By days 7 after CLI, the ratio of INBF was highest in group 1, lowest in group 2, significantly higher in group 5 than in groups 3 and 4, and significantly higher in group 4 than in group 3. Furthermore, the circulating level of CD34+ cell was highest in group 5, lowest in group 2, significantly higher in group 1 than in groups 3 and 4, and significantly higher in group 4 than in group 3.

Nitric oxide production and protein expressions of fibrotic and anti-fibrotic biomarkers in CLI area by day 14 after CLI procedure (Figure 3)

The results of Griess assay showed that the tissue level of NO in quadriceps was significantly higher in group 2 than in groups 1, 3 to 5, but it showed no difference among these four groups, suggesting an intrinsic response to ischemic stimulation in group 2 animals.

The protein expression of TGF- and phosphorylated (p)-Smad3, two indicators of fibrosis, were highest in group 2, lowest in group 1, significantly higher in group 3 than in groups 4 and 5 and significantly higher in group 4 than in group 5. Conversely, the protein expression of p-Smad1/5 and BMP-2, two indices of anti-fibrosis, displayed an opposite pattern of fibrosis among the five groups.

Protein expressions of angiogenesis factors in CLI area by day 14 after CLI procedure (Figure 4)

The protein expressions of CD31 and eNOS, two angiogenesis biomarkers, were highest in group 1, lowest in group 2, significantly higher in group 5 than in groups 3 and 4, and significantly higher in group 4 than in group 3. Additionally, the protein expression of vWF, another indicator of angiogenesis, were highest in group 5, lowest in group 2, significantly higher in groups 4 than in groups 1 and 3, and significantly higher in group 1 than in group 3. Furthermore, the protein expressions of VEGF, XCR4 and SDF-1α, three angiogenesis biomarkers, were significantly progressively increased from groups 1 to 5, suggesting an intrinsic response to ischemic stimulation further increased by HBO-EPC treatment.

Protein expression of apoptosis, mitochondrial-damage and mitochondrial integrity biomarkers in CLI area by day 14 after CLI procedure (Figure 5)

The protein expression of cleaved (c)-caspase 3, c-PARP and mitochondrial Bax, three indicators of apoptosis, and cytosolic cytochrome C, an indicator of mitochondrial-damage, were highest in group 2 and lowest in group 1, significantly higher in group 3 than in groups 4 and 5, and significantly higher in group 5 than in group 4. Conversely, the protein expression of mitochondrial cytochrome C, a mitochondrial integrity biomarker, exhibited an opposite pattern of apoptosis among the five groups.

Cellular expression of EPC biomarkers in the CLI area by day 14 after CLI procedure (Figure 6)

The cellular expression of XCR4 and SDF-1α, two indicators of EPCs, were significantly pro-
Figure 2. Ratio of ischemic to normal blood flow by laser Doppler scan measurement at days 1, 7 and 14 after CLI procedure. A. Illustrating the laser Doppler results of blood flow both hind limbs among the five groups at days 1, 7 and 14 after CLI procedure. B. Analytical result of ratio of ischemic (left limb)/normal (right limb) blood flow (INBF) at day 0, * vs. †, P < 0.0001. C. Analytical result of ratio of INBF ay day 7, * vs. other groups with different symbols (†, ‡, §, ¶), P < 0.0001. D. Analytical result of ratio of INBF ay day 14, * vs. other groups with different symbols (†, ‡, §), P < 0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 8). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). SC = sham control; CLI = critical limb ischemia; HBO = hyperbaric oxygen; EPC = endothelial progenitor cell.
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gressively increased from groups 1 to 5, implicating an intrinsic response to ischemic stimulation and further increased by HBO-EPC treatment.

Expression of endothelial cells in CLI area by day 14 after CLI procedure (Figure 7)

The expressions of CD31+ and vWF+ cells, two indicators of endothelial cells (ECs), were highest in group 1 and lowest in group 2, significantly higher in group 5 than in groups 3 and 4, and significantly higher in group 4 than in group 3.

Protein and cellular expressions of hypoxic inducible factor (HIF)-1α in CLI area by day 14 after CLI procedure (Figure 8)

The protein and cellular expressions of HIF-α were significantly progressively increased from groups 1 to 5, suggesting an intrinsic response to ischemic stimulation and notably further increased in HBO-EPC treatment.

Number of vessel density in CLI area by day 14 after CLI procedure (Figure 9)

The microscopic findings of IHC stain (i.e., α-SMA) demonstrated that the number of small vessels (i.e., ≤ 25 µM), an indicator of angiogenesis/vasculogenesis was highest in group 1, lowest in group 2, significantly higher in group 5 than in groups 3 and 4, and significantly higher in groups 4 than in group 3.

Inflammatory cellular expression in the CLI area by day 14 after CLI procedure (Figure 10)

The cellular infiltration of CD14+ and F4/80+ cells, two indicators of inflammation, were highest in group 2 and lowest in group 1, significantly lower in group 5 than in groups 3 and 4,
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In vitro study showing the HBO therapy enhanced the protein expressions of angiogenesis factors in circulatory derived ex-vivo expanded EPCs (refer to Figure S1).

Phenotype of circulatory derived EPCs at two time points of ex-vivo expanded cell culture (refer to Figure S2).

Discussion

This study, which investigated the impact of HBO-EPC therapy in a CLI setting in rats, yielded several striking implications. First, compared with SC, the circulating levels of EPCs were remarkably lower in CLI animals. However, the circulating EPC levels were remarkably increased in animals treated with HBO and more increased in HBO + EPCs, highlighting that HBO therapy augmented mobilization of EPCs from bone marrow to circulation. Second, the angiogenesis biomarkers, including those of protein and cellular expressions, were significantly higher in CLI animals treated with HBO and further significantly higher treated by HBO-EPCs than in those of CLI animals treated with culture medium, suggesting HBO treatment not only systemically enhanced but also locally (i.e., in ischemic zone) enhanced angiogenesis factors. Third, as compared with the CLI-EPC group, the number of EPCs in the CLI area was and significantly lower in group 4 than in group 3.

Figure 4. Protein expressions of angiogenesis factors in the CLI area by day 14 after the CLI procedure. A. Protein expression of CD31, * vs. other groups with different symbols (†, †, ‡, §, ¶), P < 0.0001. B. Protein expression of endothelial nitric oxide synthase (eNOS), * vs. other groups with different symbols (†, †, ‡, §, ¶), P < 0.0001. C. Protein expression of von Willebrand factor (vWF), * vs. other groups with different symbols (†, †, ‡, §, ¶), P < 0.0001. D. Protein expression of vascular endothelial growth factor (VEGF), * vs. other groups with different symbols (†, †, ‡, §, ¶), P < 0.0001. E. Protein expression of CXCR4, * vs. other groups with different symbols (†, †, ‡, §, ¶), P < 0.0001. F. Protein expression of stromal-cell derived factor (SDF)-1α, * vs. other groups with different symbols (†, †, ‡, §, ¶), P < 0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6). Symbols (*, †, †, ‡, §, ¶) indicate significance (at 0.05 level). SC = sham control; CLI = critical limb ischemia; HBO = hyperbaric oxygen; EPC = endothelial progenitor cell.
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significantly increased in the CLI-HBO-EPC group, suggesting that HBO therapy could enhance the EPC expression in CLI ischemic zone. Fourth, as compared with CLI animals, the blood flow in the CLI area was markedly increased in CLI animals with HBO or EPC treatment and further markedly increased in CLI animals with HBO-EPC treatment, highlighting the synergic effect of combining these two therapeutic regimens.

HBO therapy was identified a long time ago as an effective alternative method for patients with chronic ischemic PAOD [11]. In addition, EPC therapy has previously been established as a safe and promising for treatment for limb ischemia [17, 24]. An essential finding in the present study was that the blood flow significantly increased in CLI animals with HBO or EPC treatment as compared with those of CLI only animals. Additionally, when we looked at the effect of restoring the blood flow in the CLI area, we identified HBO therapy was comparable with that of EPC therapy. Our findings are, therefore, consistent with the findings of previous studies [11, 17, 24]. The most important finding in the present study was that the combined HBO-EPC treatment was superior to either one alone for restoring the blood flow in CLI region. Accordingly, our findings in addition to extending the findings of the previous studies [11, 17, 24], highlight the potential role of combined HBO-EPC therapy for those patients who have CLI and respond poorly to traditionally therapeutic methods.

Interestingly, when we carefully reviewed the literature, we found that HBO therapy was not
effective at salvaging patient’s progressive limb ischemia [13], suggesting that due to the diffusion of oxygen into the ischemic area/organ creating a hyperbaric environment is extremely limited [14, 15]. On the other hand, intrinsic response of pathophysiologic alarming for EPC homing to ischemic area has been established to often be insufficient due to severe ischemia and extremely hypoxic situation [17]. A principal finding in the present study was that the protein levels of SDF-1α and HIF-1α were notably higher in CLI animals as compared with SC animals. Additionally, the generation of NO in the ischemic area was also remarkably increased in CLI animals in comparison with in animals in other groups. How-

**Figure 6.** Cellular expressions of angiogenesis in CLI area by day 14 after CLI procedure. A-E. Illustration of immunofluorescence (IF) microscopy findings (400 ×) for identification of SDF-1α+ cells (green color). Red color indicates Dil-dye stained EPCs. F. Analytical result of number of SDF-1α+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P < 0.0001. G-K. IF microscopy findings (400 ×) for identification of CXCR4+ cells (green color). Red color indicated Dil-dye stained EPC). L. Analytical result of number of CXCR4+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P < 0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). SC = sham control; CLI = critical limb ischemia; HBO = hyperbaric oxygen; EPC = endothelial progenitor cells.
ever, the blood flow in the CLI zone was extremely limited in these CLI animals, implicating the intrinsic response for restoring the blood flow was still incompetent.

On the other hand, the protein expressions of SDF-1α and HIF-1α were remarkably enhanced in HBO and EPC therapy and more remarkably enhanced in HBO-EPC therapy as compared with CLI animals. Additionally, the circulating levels (i.e., Sca-1/CD31+, C-kit/CD31+, CD34+) and tissue level (i.e., CXCR4+, SDF-1+) of EPCs were markedly increased in HBO and EPC therapy and more remarkably increased in HBO-EPC therapy as compared with CLI animals. Our findings, therefore, suggested that further...
increased EPCs in CLI area could be due to the results of increased protein levels of SDF-1α and HIF-1α in CLI zone (i.e., two chemokine for EPC homing) after HBO therapy. Interestingly, previous studies have shown that SDF-1α and HIF-1α augmented angiogenesis in ischemic tissue/organ mainly through increasing the EPC mobilization into circulation and homing into ischemic area [24, 29]. Additionally, HBO therapy involving improving chronic ischemic PAOD has been proposed to be mainly through an increase of vascular wall permeability and pro-

Figure 8. Protein and cellular expressions of hypoxic inducible factor (HIF)-1α by day 14 after CLI procedure. A. Protein and cellular expressions of HIF-1α, * vs. other groups with different symbols (†, ‡, §, ¶), P < 0.0001. B-F. Illustration of immunofluorescence (IF) microscopic findings (400 ×) for identification of HIF-1α+ cells (green color). Red color indicated Dil-dye stained ECs. G. Analytical result of number of HIF-1α+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P < 0.0001.

Figure 9. Microscopic findings for identification of number of small vessel density in CLI area by day 14 after CLI procedure. A-E. Illustration of the microscopic finding (200 ×) for identification of α-smooth muscle actin (α-SMA)+ stained small vessels (≤ 25 μM > 10 μM) (gray color). F. Analysis of number of small vessels, * vs. other groups with different symbols (†, ‡, §, ¶), P < 0.001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 9). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). HPF = high-power field. SC = sham control; CLI = critical limb ischemia; HBO = hyperbaric oxygen; EPC = endothelial progenitor cells.

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ductions of HIF-1α and SDF-1α that in turn, enhances angiogenesis and blood flow in the ischemic area [12]. Accordingly, our findings, in addition to supporting the findings of previous studies [12, 24, 29], could also explain the results of further enhancing the blood flow in the CLI area after combined HBO-EPC treatment was due to a synergic effect of this combined therapy.

Previous studies have shown that EPC therapy improved the endothelial biomarkers/function and number of small vessels in the ischemic region [24, 29]. Another important finding in the present study was that the levels of eNOS (i.e., protein level) and the levels of CD31 and vWF (i.e., cellular level), three indicators of EC integrity, were substantially higher in CLI animals with HBO or EPC therapy and further substan-

Figure 10. Inflammatory cellular expressions in CLI area by day 14 after CLI procedure. A-E. Illustration of immunofluorescence (IF) microscopy findings (400 ×) for identification of CD14+ cells (green color). Red color indicates Dil-dye stained ECs. F. Analysis of number of CD14+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P < 0.0001. G-K. IF microscopy findings (400 ×) for identification of F4/80+ cells (green color). Red color indicates Dil-dye stained ECs. L. Analysis of number of F4/80+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P < 0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6). Symbols (*, †, ‡, §, ¶) indicate significance at 0.05 level. SC = sham control; CLI = critical limb ischemia; HBO = hyperbaric oxygen; EPC = endothelial progenitor cell.
tially higher in CLI with combined HBO-EPC therapy than in those of CLI only animals. Likewise, the number of small vessels also expressed a similar pattern to these angiogenesis markers among the groups of animals. Our findings, in addition to reinforcing the findings of previous studies [24, 29], once again explained why the blood flow was significantly increased in HBO- or EPC-treated CLI animals and further increased in combined HBO-EPC treated CLI animals than in CLI only animals.

Organ ischemia/damage always elicits an inflammatory reaction and upregulation of apoptosis, fibrosis and mitochondrial damage [9, 24, 28-32]. Another essential finding in the present study was that the fibrotic, apoptotic, inflammatory and mitochondrial-damaged biomarkers were notably increased in CLI animals in comparison with SC animals and were notably reduced in HBO-treated or in EPC-treated animals and further notably reduced in combined HBO-EPC-treated animals. Our findings, in addition to being consistent with the findings of previous studies [9, 24, 28-32], suggest that these therapeutic regimens protected the quadriceps damage against CLI via the restoring the blood flow in the ischemic region.

Study limitations

This study has limitations. First, we did not test the optimal dose of HBO and EPC for the CLI setting in rats. Therefore, we did not provide information about whether the effect of EPC therapy for improving the blood flow in CLI was superior to HBO or vice versa. Second, this study did not prove whether HBO therapy could increase the vascular wall permeability in the CLI setting in rats. Third, this study did not assess the angiogenic factors, chemokines and inflammatory markers in the plasma samples. Therefore, how much contribution of HBO to the ischemia recovery in rodents is currently uncertain. Finally, inhibiting VEGF in vivo was not performed in the current study. Accordingly, the underlying mechanism by which HBO acts on which downstream signaling pathway is still not clear.

In conclusion, HBO-EPC therapy has a synergic effect improving the blood flow in the CLI setting mainly through upregulation of chemokines and angiogenesis factors at both the cellular and protein levels.

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Disclosure of conflict of interest

None.

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Figure S1. HBO therapy for 3 h enhanced the protein expressions of angiogenesis factors in circulatory derived ex-vivo expanded EPCs. (A) Protein expression of von Willebrand factor (vWF), * vs. †, p < 0.01. (B) Protein expression of vascular endothelial growth factor (VEGF), * vs. †, p < 0.05. (C) Protein expression of stromal cell-derived growth factor (SDF)-1α, * vs. †, p < 0.05. (D) Protein expression of CD31, * vs. †, p < 0.01. (E) Protein expression of endothelial nitric oxide synthase (eNOS), * vs. †, p < 0.05. (F) Protein expression of transforming growth factor (TGF)-β, * vs. †, p < 0.005. (G) Protein expression of hepatocyte growth factor (HGF), * vs. †, p = 0.01. n = 4 in each group. HBO = hyperbaric oxygen; EPC = endothelial progenitor cell.
Figure S2. Phenotype of circulatory derived EPCs at two time points (i.e., at days 7 and 21 after cell culturing) of ex-vivo expanded cell culture. Microscopic findings (i.e., 40 ×, 100 ×, 200 ×, 400 ×) illustrating the cobblestone-like appearance of ex-vivo expanded culturing cells, i.e., indicating a morphological feature of EPC phenotype, was observed at days 7 and 21 of cell-culturing time points. EPC = endothelial progenitor cell.