# Benefit of combined extracorporeal shock wave and bone marrow-derived endothelial progenitor cells in protection against critical limb ischemia in rats*

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*Objectives:* We hypothesized that combined treatment with extracorporeal shock wave and bone marrow-derived endothelial progenitor cells might exert enhanced protection against critical limb ischemia in rats.

*Methods:* Male Sprague-Dawley rats (n = 9 for laser Doppler study and n = 6 for laboratory examinations in each group) were divided into group 1 (sham control), group 2 (critical limb ischemia treated with culture medium), group 3 (critical limb ischemia treated with intramuscular bone marrow-derived endothelial progenitor cells [$2.0 \times 10^6$ cells]), group 4 (critical limb ischemia treated with extracorporeal shock wave [280 impulses at 0.1 mJ/mm²]), and group 5 (combined bone marrow-derived endothelial progenitor cell-extracorporeal shock wave) after critical limb ischemia induction.

*Results:* By day 21, laser Doppler showed substantially lower ratios of ischemic/normal blood flow in group 2 compared with other groups ($p < .001$). The protein expressions of mitochondrial cytochrome c, stromal cell-derived factor-1, C-X-C chemokine receptor type 4, vascular endothelial growth factor, and endothelial nitric oxide synthase were remarkably higher in group 5 than

in groups 2 to 4, and notably higher in groups 3 and 4 than in group 2 (all $p < .01$). The messenger RNA expressions of proinflammatory and apoptotic biomarkers and oxidative stress were reduced in group 5 compared with groups 2 to 4, and notably lower in groups 3 and 4 than in group 2 (all $p < .01$). The messenger RNA expressions of anti-inflammatory and antiapoptotic biomarkers were lower in group 2 than in other groups (all $p < .01$). Immunofluorescent staining showed higher numbers of CD31+ stromal cell-derived factor-1+, chemokine receptor type 4+, and von Willebrand factor+ cells, and vessels in the ischemic area in group 5 than in groups 2 to 4, and in groups 3 and 4 than in group 2 (all $p < .04$).

*Conclusion:* Combined treatment with bone marrow-derived endothelial progenitor cells and extracorporeal shock wave is superior to either bone marrow-derived endothelial progenitor cells or extracorporeal shock wave alone in improving ischemia in rodent critical limb ischemia. (Crit Care Med 2012; 40:169–177)

KEY WORDS: angiogenesis; critical limb ischemia; endothelial progenitor cells; extracorporeal shock wave

Peripheral arterial disease is a highly prevalent atherosclerotic syndrome that affects approximately 8 to 12 million individuals in the United States and is associated with significant morbidity and mortality (1). Importantly, patients with peripheral arterial disease may develop critical limb ischemia (CLI) at a later stage of the disease (1, 2).

The pathophysiological consequences of CLI is initiated by endothelial dysfunction accompanied by upregulated inflammatory mediators, which facilitates leukocyte-endothelial interactions, vascular smooth muscle cell proliferation, atherosclerotic formation and propagation, and finally, development of obliterating atherosclerosis. As a consequence, the peripheral perfusion is critically reduced due to macro and microcirculatory alterations (1, 3–9).

Although bypass surgical intervention is the standard method with the highest successful rate, the procedural failure rate, and short-term and long-term untoward clinical outcomes in some patients remains problematic. Development of a more cost-effective means to treat patients with CLI and those unsuitable for either surgical or percutaneous intervention, therefore, is obligatory and urgently needed.

Transplantation of endothelial progenitor cell (EPCs) has been shown to be

effective in improving ischemia-related organ dysfunction through enhanced angiogenesis/vasculogenesis (10–12). Recent studies have further demonstrated that combined therapy using gene-modified or viral-transfected overexpression of chemokines and EPCs would offer additional benefits in the setting of CLI (13). However, these studies (13) were experimental rather than clinical because of the associated ethical issues, including the unknown impact of introducing novel genetic materials into the human body and the risk of potential viral infection. Therefore, a less ethically controversial clinical strategy in cell therapy applicable to daily clinical practice is the current direction of research. Interestingly, recent studies (14, 15) have demonstrated that shock wave (SW) therapy increased angiogenesis/vasculogenesis, which in turn improved ischemia-related organ dysfunction (16, 17). However, a therapeutic effect of SW in the setting of CLI has seldom been reported (18). Furthermore, whether combined therapy with bone marrow-derived EPCs (BMDEPCs) and SW offers additional benefit for CLI has not been demonstrated. Accordingly, the purpose of this study was to evaluate whether a combined therapy using BMDEPCs and extracorporeal SW (ECSW) is superior to either one alone in improving CLI in rats. Such a therapeutic proposal was based on two reasons: 1) demonstration of incorporation of transplanted EPCs into growing collateral vasculature in the CLI area (19) to enhance angiogenesis; and 2) our hypothesis of possible SW-induced enhancement of local chemokine production (i.e., stromal cell-derived factor [SDF]-1α and vascular endothelial growth factor [VEGF]) that might play an essential role in EPC homing to ischemic areas for neovascularization.

## MATERIALS AND METHODS

*Ethics.* All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at our institute and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

*Animal Model of CLI.* Twelve-week-old male adult Sprague-Dawley rats (n = 45), weighing 300–325 g, (Charles River Technology, BioLASCO Taiwan, Taiwan), were divided (n = 9 for laser Doppler study and n = 6 for laboratory examinations in each group) into group 1 (sham control), group 2 (CLI treated with culture medium), group 3 (CLI treated
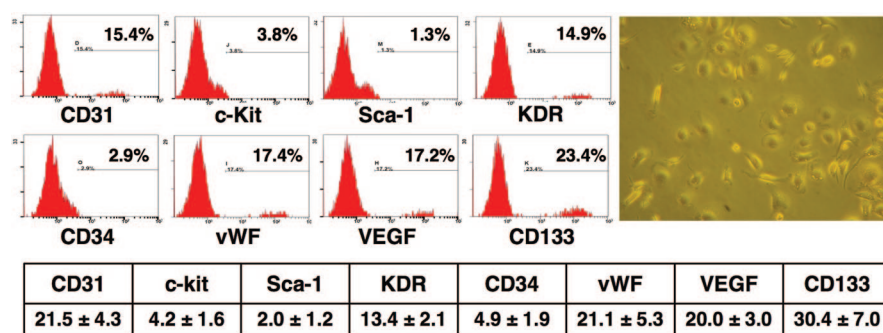


Figure 1. *Left Upper Panel* shows the results of bone marrow-derived endothelial progenitor cells in one rat after 21 days of cell culture using flow cytometry. *Right Upper Panel* shows spindle-shaped and cobblestone-like morphology typical of endothelial cells observed under the microscope (×200) following 21-day culture in M199 culture medium in the same rat. *Lower Panel*: the mean percentage of endothelial progenitor cells after 21-day cell culture (n = 3). *Sca-1*, stem cell antigen-1; *KDR*, kinase insert domain receptor; *vWF*, von Willebrand factor; *VEGF*, vascular endothelial growth factor.
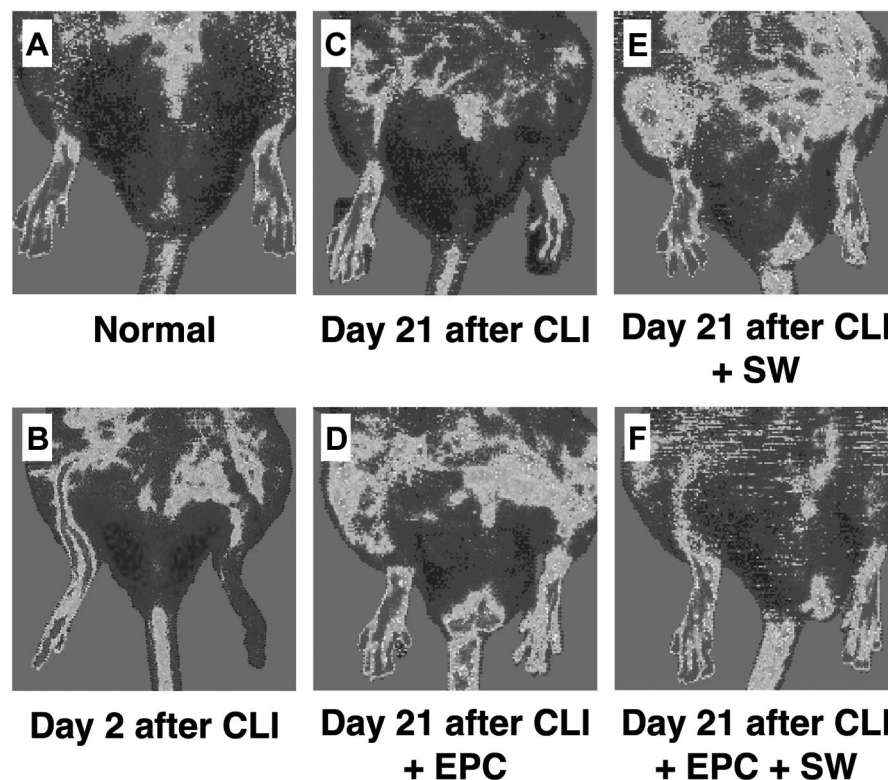
| CD31 | c-kit | Sca-1 | KDR | CD34 | vWF | VEGF | CD133 |
|---|---|---|---|---|---|---|---|
| 21.5 ± 4.3 | 4.2 ± 1.6 | 2.0 ± 1.2 | 13.4 ± 2.1 | 4.9 ± 1.9 | 21.1 ± 5.3 | 20.0 ± 3.0 | 30.4 ± 7.0 |



Figure 2. Illustration of laser Doppler scanner on day 21 after critical limb ischemia (*CLI*). *A*, Normal blood flow in both hind limbs of normal control rat. *B*, The blood flow was markedly reduced over the left hind limb on day 2 after CLI. This finding indicated the CLI model was successfully created. By day 21 after CLI procedure, the laser Doppler scan showing the blood flow was substantially reduced in CLI rats without treatment (*C*) than in rats with CLI + shock wave (*SW*) (*D*), CLI + endothelial progenitor cell (*EPC*) (*E*), or combined EPC and SW therapy (*F*).

with intramuscular injection of BMDEPCs [2.0 × 10⁶ cells]), group 4 (CLI treated with ECSW [280 impulses at 0.1 mJ/mm²]), and group 5 (combined BMDEPCs and ECSW). The energy dosage of ECSW (280 impulses at 0.1 mJ/mm²) utilized in this study was based on our recent reports (15, 20), with a modified energy dosage described in detail in the supplemental information (see Supplemental Fig. 1 [Supplemental Digital Content 1, http://

links.lww.com/CCM/A290]; and its legend [Supplemental Digital Content 2, http://links.lww.com/CCM/A291]). The number of EPCs used in the present study was in accordance with another study of ours (21).

Rats in groups 2 to 5 were anesthetized by intraperitoneal injections of chloral hydrate (35 mg/kg). The rats were placed in a supine position on a warming pad at 37°C with the left hind limbs shaved. Under sterile condi-

tions, the left femoral artery, small arterioles, and circumferential femoral artery were exposed and ligated over their proximal and distal portions and then were removed. To avoid the presence of collateral circulation, the branches were removed together. However, the veins were left intact during the procedure.

*Isolation of BMD Mononuclear Cells and Differentiation into EPCs.* The protocols on isolation and culture of BMD mononuclear cells were based on our recent study (22). Briefly, on day 21 before CLI induction, pathogen-free Sprague-Dawley rats (n = 12) were anesthetized by intraperitoneal injections of chloral hydrate (35 mg/kg). Bone marrow was carefully aspirated from both distal femoral bones for each animal, followed by isolation of BMD mononuclear cells (approximately $1.0 \times 10^6$) by Ficoll-paque (Amersham) density-gradient centrifugation. These cells were then cultured in differential endothelial cell culture medium for 21 days and plenty ($\sim 2.0 \times 10^6$) of endothelial cells were found attached on the plate, i.e., BMDEPCs (Fig. 1).

*Flow Cytometric Quantification of EPCs Based on Surface Markers.* To identify the population of EPCs following 21-day cell culture, the cells were trypsinized, washed twice with phosphate buffered saline (PBS), and immunostained for 30 mins on ice with the following antibodies: phycoerythrin-conjugated antibodies against CD133 (BD Pharmingen), stem cell antigen-1 (BD Pharmingen), and CD34 (BD Pharmingen); fluorescein isothiocyanate-conjugated antibodies against c-kit (BD Pharmingen); monoclonal antibodies against CD31 (Abcam) and VEGF (Abcam); and polyclonal antibodies against von Willebrand factor (vWF) (Millipore) and kinase insert domain receptor (Thermo). Cells labeled with nonfluorescence-conjugated antibodies were further incubated with Alexa Fluor 488-conjugated antibodies specifically against mouse or rabbit immunoglobulin G (Invitrogen). Isotype-identical antibodies (immunoglobulin G) served as controls. Flow cytometric analyses were performed by utilizing a fluorescence-activated cell sorter (Beckman Coulter FC500 flow cytometer) (Fig. 1, relevant description in the result section).

*Autologous EPC Implantation and Application of ECSW.* BMDEPCs ($2.0 \times 10^6$) were labeled by CM-DiI (Vybrant Dil cell-labeling solution, Molecular Probes) 30 mins before CLI induction and then injected intramuscularly into the hind limb ischemic area once before animal recovery from anesthesia.

ECSW was applied to the CLI area of rats in groups 3 and 5 under anesthetized conditions, and on day 2 after the procedure. The procedures of ECSW therapy were as follows: conduction jelly was first applied to the shaved ischemic area of left leg on which an energy probe (R05, HealthTronics, Switzerland) was placed and focused on the ischemic area; a total of 280 shots were then applied to the site
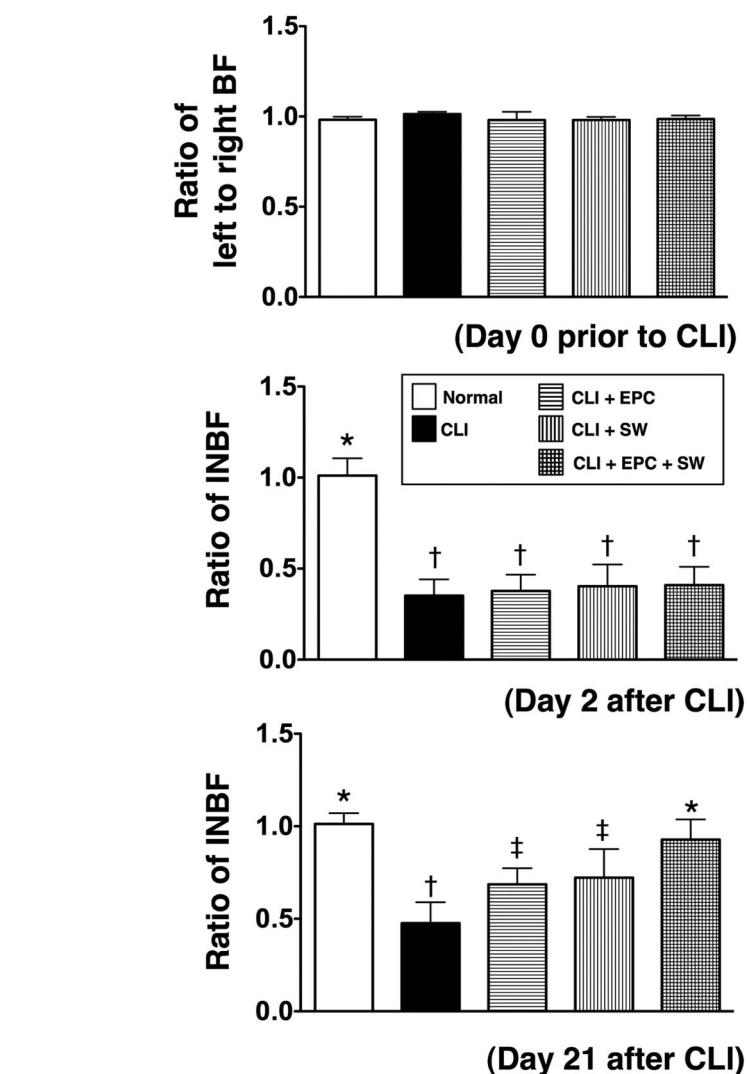


**Figure 3.** Ratio of ischemic/normal blood flow (*INBF*) by laser Doppler scan on days 2 and 21 after critical limb ischemia (*CLI*) procedure (n = 9). *Upper Panel*: Ratio of INBF was similar in the five groups of animals. *Middle Panel*: Ratio of INBF was notably lower in groups of CLI, CLI + endothelial progenitor cell (*EPC*), CLI + shock wave (*SW*), and CLI + combined EPC and SW than in normal control on day 2 after CLI procedure. * vs. †, $p < .001$. *Lower Panel*: Ratio of INBF was notably lower in the CLI group than in other groups, lower in groups of CLI + EPC and CLI + SW than normal control and CLI + combined EPC and SW group on day 21 after procedure. A similar ratio of INBF between normal control and CLI + combined EPC and SW groups, and similar between CLI + EPC and CLI + SW groups on day 21 after CLI procedure. Statistical analysis by one-way analysis of variance. * vs. other groups, $p < .0001$. Symbols (*, †, ‡) indicate significant difference (at .05 level) by Tukey multiple comparison procedure.

of arterial injury within 90 secs (i.e., two shots per second).

*Measurement of Blood Flow With Laser Doppler (Fig. 2).* Rats were anesthetized by intraperitoneal injections of chloral hydrate (35 mg/kg) before CLI induction, and at days 2 and 21 after CLI induction before being killed (n = 9 for each group). The rats were placed in a supine position on a warming pad at 37°C and the blood flow was detected in both inguinal areas by a laser Doppler scanner (moorLDLS, Moor, UK) (Fig. 2, detailed description in the result section). The ratio of flow in the left (ischemic) leg and right (normal) leg was computed. The rats were killed

and the quadriceps muscle was collected for individual study.

*Western Blot Analysis.* Equal amounts (10–30 μg) of protein extracts from ischemic quadriceps of the animals (n = 6 for each group) were loaded and separated by SDS-PAGE using 12% acrylamide gradients. The membranes were incubated with monoclonal antibodies against vascular cell adhesion molecule chemokine receptor type 4 (1:1000, Abcam), VEGF (1:1000, Abcam), SDF-1α (1:1000, Cell Signaling), connexin 43 (1:2000, Chemicon), cytochrome c (1:2000, BD), endothelial nitric oxide synthase (1:1000, Abcam), Bax (1:1000, Abcam), and Bcl-2 (1:200, Abcam). Sig-

nals were detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit immunoglobulin G. The Oxyblot Oxidized Protein Detection Kit was purchased from Chemicon (S7150) for oxyblot protein analysis. Proteins were transferred to nitrocellulose membranes which were then incubated in the primary antibody solution (anti-DNP 1:150) for 2 hrs, followed by incubation with the secondary antibody solution (1:300) for 1 hr at room temperature. The washing procedure was repeated eight times within 40 mins. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences) which was then exposed to Biomax L film (Kodak). For quantification, enhanced chemiluminescence signals were digitized using Labwork software (UVP). For oxyblot protein analysis, a standard control was loaded on each gel.

*Real-Time Quantitative Polymerase Chain Reaction Analysis.* Real-time polymerase chain reaction was conducted (n = 6 for each group) using LightCycler TaqMan Master (Roche, Germany) in a single capillary tube according to the manufacturer's guidelines for individual component concentrations as we previously reported (20, 22).

*Immunofluorescence (IF) Staining.* IF staining was performed for the examinations of CD31+ and vWF+ cells (n = 6 for each group) using respective primary antibodies based on our recent study (22). Irrelevant antibodies were used as controls in the current study.

*Vessel Density in Limb Ischemic Area.* Immunohistochemical staining of blood vessels was performed (n = 6 for each group) with α-smooth muscle actin (1:400) as the primary antibody at room temperature for 1 h, followed by washing with PBS thrice. Ten minutes after the addition of the anti-mouse horseradish peroxidase-conjugated secondary antibody, the tissue sections were washed with PBS thrice. Then, 3,3′ diaminobenzidine (0.7 g/tablet) (Sigma) was added, followed by washing with PBS thrice after 1 min. Finally, hematoxylin was added as a counterstain for nuclei, followed by washing twice with PBS after 1 min. Three sections of quadriceps were analyzed in each rat. For quantification, three randomly selected high-power fields (×100) were analyzed in each section. The mean number per high-power field for each animal was then determined by summation of all numbers divided by nine.

*Statistical Analysis.* Quantitative data are expressed as means ± SD. Statistical analysis was adequately performed by analysis of variance followed by Tukey multiple comparison procedure. Statistical analysis was performed using SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC). A probability value <.05 was considered statistically significant.
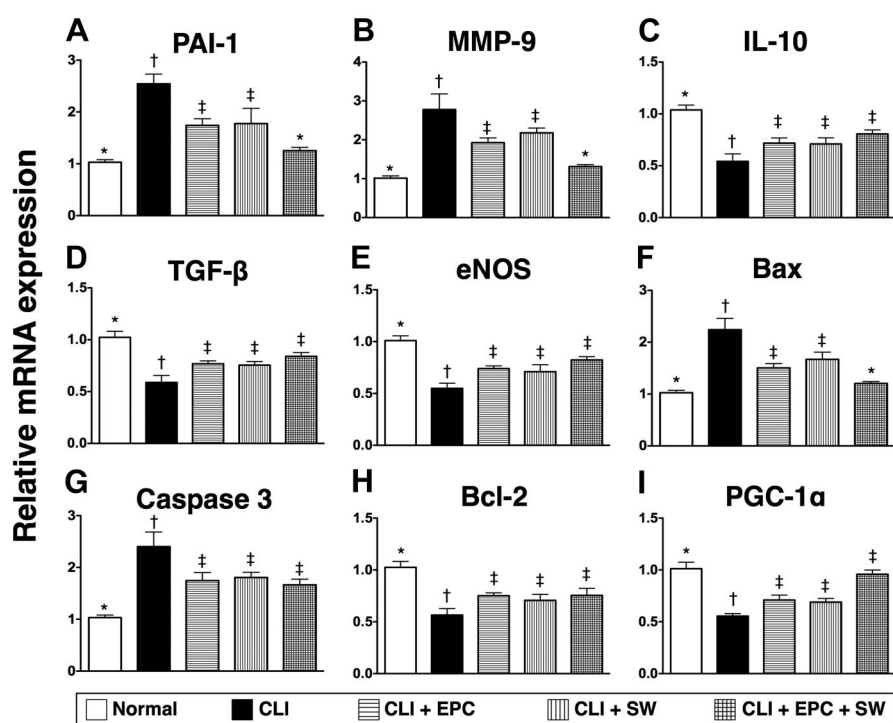


Figure 4. Real-time polymerase chain reaction of ischemic skeletal muscle on day 21 following critical limb ischemia (*CLI*) induction (n = 6). *A*, Plasminogen activator inhibitor (*PAI*)-1 mRNA expression; *B*, Matrix metalloproteinase (*MMP*)-9 mRNA expression; *C*, Interleukin (*IL*)-10 mRNA expression; *D*, Transforming growth factor (*TGF*)-β mRNA expression; *E*, Endothelial nitric oxide synthase (*eNOS*) mRNA expression; *F*, Bax mRNA expression; *G*, Caspase 3 mRNA expression; *H*, Bcl-2 mRNA expression; *I*, Peroxisome proliferator-activated receptor-γ coactivator-1α (*PGC-1α*) mRNA expression. Symbols (*, †, ‡) indicate significant difference (at .05 level) by Tukey multiple comparison procedure. *SW*, shock wave; *EPC*, endothelial progenitor cell.

## RESULTS

*Flow Cytometric Analysis (Fig. 1).* The most prevalent BMDEPC marker was CD133, followed by CD31, vWF, VEGF, and kinase insert domain receptor, whereas CD34, c-kit, and stem cell antigen-1 were the least expressed at day 21 of cell culture.

*Laser Doppler Analysis of Blood Flow (Figs. 2 and 3).* The ratios of ischemic/normal blood flow (INBF) did not differ among the five groups (n = 9 for each group), (i.e., normal control [group 1], CLI only [group 2], CLI + EPC [group 3], CLI + SW [group 4], and CLI + EPC + SW [group 5]) on day 0 before the CLI procedure (Fig. 3). There was also no significant difference among groups 2 to 5, but it was markedly reduced in groups 2 to 5 compared with group 1 on day 2 after the CLI procedure. By postoperative day 21, the ratio of INBF was significantly reduced in group 2 than in other groups, notably reduced in groups 3 and 4 than in groups 1 and 5, but did not differ between groups 1 and 5 or between groups 3 and 4 (Figs. 2 and 3).

*mRNA Expressions of Inflammatory, Apoptotic, and Mitochondrial Functional Markers in Ischemic Quadriceps on Day 21 After CLI Procedure (Fig. 4).* The mRNA expressions of plasminogen activator inhibitor-1 and matrix metalloproteinase-9, two inflammatory biomarkers, were notably higher in group 2 than in other groups, lowest in groups 1 and 5, but showed no difference between groups 3 and 4 or between groups 1 and 5. In contrast, the mRNA expressions of interleukin-10 and transforming growth factor-β, two indexes of anti-inflammation, and endothelial nitric oxide synthase, an indicator of anti-inflammation as well as an index of angiogenesis, were notably lower in group 2 than in other groups, and significantly reduced in groups 3 to 5 as compared to that in group 1, but there were no notable difference among groups 3 to 5.

The mRNA expressions of Bax and caspase 3, two indicators of apoptosis, were remarkably higher in group 2 than in other groups. Additionally, Bax mRNA expression was significantly higher in
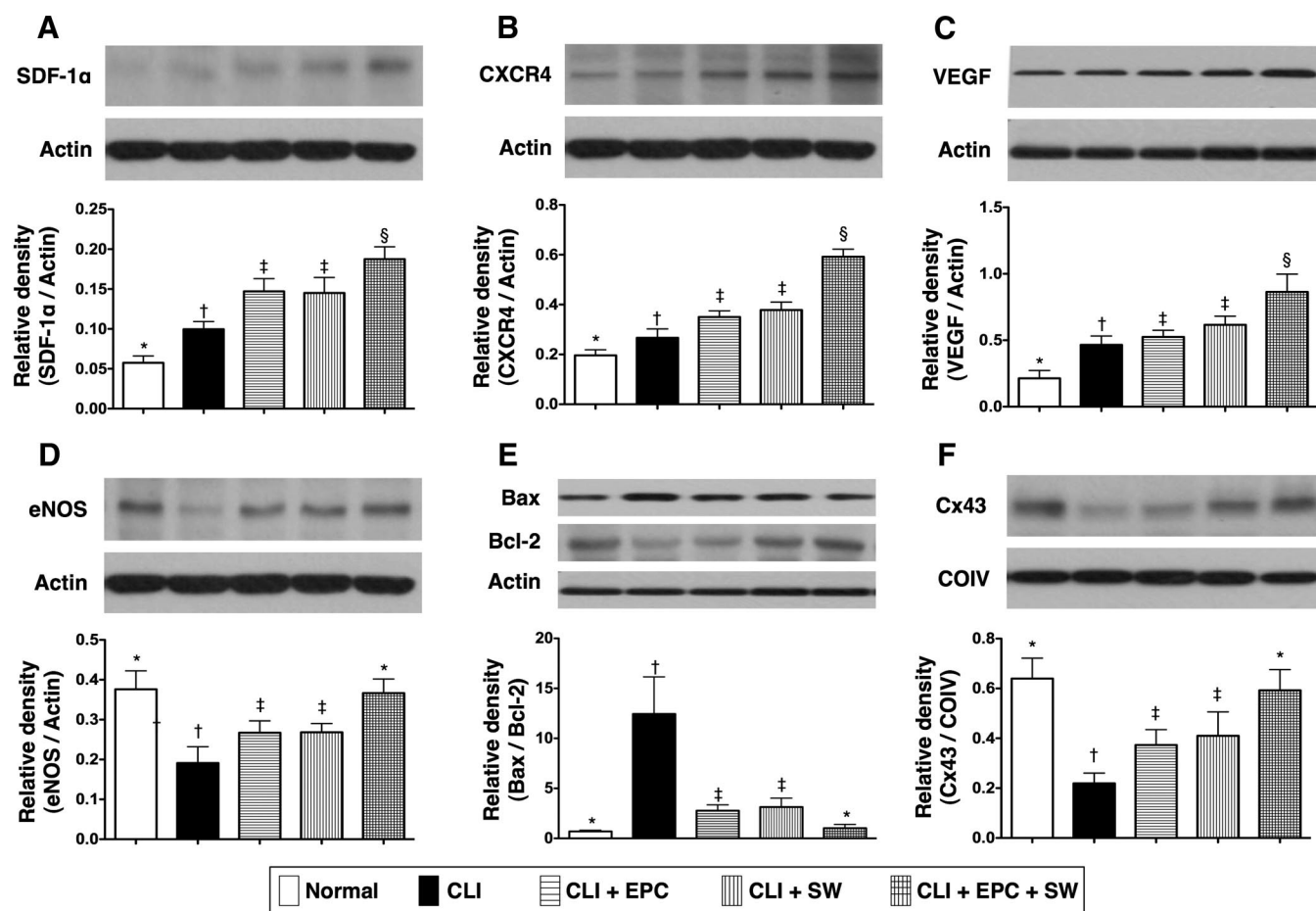
Figure 5. The Western blot results of ischemic skeletal muscle on day 21 following critical limb ischemia (*CLI*) induction (n = 6). *A*, Protein expression of stromal cell-derived factor (*SDF*)-1α; *B*, Protein expression of chemokine receptor type 4 (*CXCR4*); *C*, Protein expression of vascular endothelial growth factor (*VEGF*); *D*, Protein expression of endothelial nitric oxide synthase (*eNOS*); *E*, Relative ratio of protein expression of Bax to Bcl-2; *F*, Protein expression of connexin 43 (*Cx43*). Symbols (*, †, ‡, §) indicate significant difference (at .05 level) by Tukey multiple comparison procedure. *COIV*, cytochrome c oxidase subunit IV; *EPC*, endothelial progenitor cell; *SW*, shock wave.

groups 3 and 4 than in groups 1 and 5, but did not differ between groups 1 and 5 or between groups 3 and 4. Furthermore, no significant difference was noted in caspase 3 mRNA expression among groups 3 to 5. However, it was remarkably higher in groups 3 to 5 than in group 1. Conversely, the Bcl-2 mRNA expression, an index of antiapoptosis, was significantly lower in group 2 than in other groups, and significantly lower in groups 3 to 5 than in group 1, but did not differ among groups 3 to 5.

The mRNA expression of peroxisome proliferator-activated receptor-γ coactivator-1α, a transcriptional coactivator for regulating mitochondrial metabolism and biogenesis, was notably reduced in group 2 compared with that in other groups, and lowest in groups 1 and 5, but did not differ between groups 3 and 4 or between groups 1 and 5.

*The Protein Expressions of Ischemic Quadriceps on day 21 after CLI Proce-*dure (Figs. 5 and 6). The protein expressions of SDF-1α, chemokine receptor type 4, VEGF, and endothelial nitric oxide synthase, indicators of angiogenesis/vasculogenesis, were substantially lower in groups 1 and 2 than in groups 3, 4, and 5, and remarkably reduced in groups 3 and 4 compared with that in group 5, but was similar between groups 3 and 4 (Fig. 5, *A-D*). These findings imply that enhanced productions of these angiogenesis markers only occur in conditions of ischemic stress, notably reinforced in response to either BMDEPCs or ECSW therapy, and further substantially augmented by combined therapy with BMDEPCs and ECSW. Furthermore, the ratio of Bax-to-Bcl-2 protein expression was substantially higher in group 2 than that in groups 3, 4, and 5, and significantly higher in groups 3 and 4 compared to that in group 5, but did not differ between group 3 and group 4 (Fig. 5*E*).

The protein expression of connexin 43, an indicator of the integrity of cellular signal transduction and communication between skeletal muscle cells, was significantly lower in group 2 than in other groups, and highest in groups 1 and 5, but was similar between groups 3 and 4, and between groups 1 and 5 (Fig. 5*F*).

The protein expressions of mitochondrial cytochrome c was significantly lower in group 2 than in other groups, highest in groups 1 and 5, but did not differ between groups 3 and 4 or between groups 1 and 5 (Fig. 6*A*). Conversely, the cytosolic expression of this protein was significantly enhanced in group 2 compared with that in other groups, lowest in groups 1 and 5, but did not differ between groups 3 and 4, or between groups 1 and 5 (Fig. 6*B*). These findings indicate that the expression of cytochrome c, an index of energy supply in mitochondria, was more preserved in group 5 than in groups
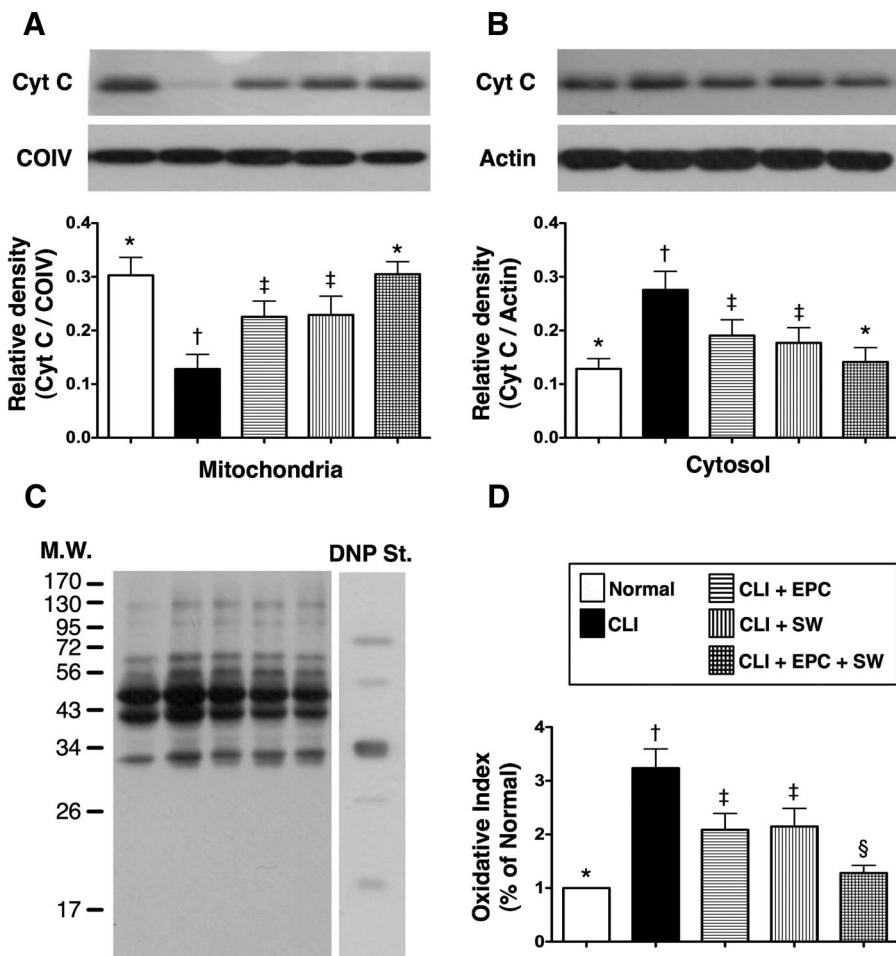
Figure 6. *A*, Mitochondrial cytochrome c (*Cyt c*) protein expression in ischemic area; *B*, Cytosolic Cyt c protein expression; *C* and *D*, Oxidative index, protein carbonyls. n = 6 for each group. Symbols (*, †, ‡, §) indicate significant difference (at .05 level) by Tukey multiple comparison procedure. *CLI*, critical limb ischemia; COIV, cytochrome c oxidase subunit IV; DNP St, 1-3 dinitrophenylhydrazone standard; *EPC*, endothelial progenitor cell; *SW*, shock wave.
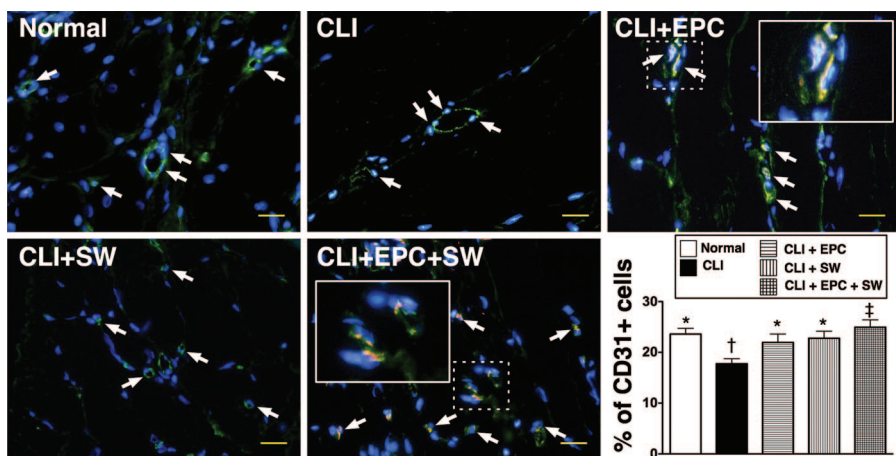


Figure 7. Immunofluorescence microscope (×400) findings of CD31+ cells, an indicator of endothelial progenitor cells (*EPCs*), in ischemic area of each group (n = 6) on day 21 following critical limb ischemia (*CLI*) procedure. The number of CD31+ cells (*white arrows*) (identification by blue color [DAPI for nuclei] and green color [CD31+ staining]) was remarkably lower in the CLI group than in other groups. Conversely, the number of CD31+ cells was substantially higher in the CLI + EPC + shock wave (*SW*) group than in other groups. The *square box* shows the double stain of CD31+ EPC (Dil dye and CD31+ staining) under high magnification of the *dotted box* in the CLI + EPC and CLI + EPC + SW groups, indicating these cells were derived from implanted EPCs after 21-day cell culture. Symbols (*, †, ‡) indicate significant difference (at .05 level) by Tukey multiple comparison procedure. The scale bars in right lower corner represent 20 μm.

2 to 4. The increase in cytosolic cytochrome c content also suggests significant mitochondrial damage with cytochrome c release into the cytosol in the quadriceps of group 2 animals. Similarly, the protein expression of oxidative stress was remarkably increased in group 2 than in other groups, and was lowest in groups 1 and 5 (Fig. 6, *C* and *D*), but was similar between groups 3 and 4, and also between groups 1 and 5.

*Identification of Implanted BMDEPCs in Ischemic Area (Figs. 7 and 8).* By day 21 following CLI induction, the rats in groups 3 and 5 were killed for identifying BMDEPCs in the ischemic area. Numerous CM-DiI-stained BMDEPCs were found to have engrafted into vessels and ischemic tissue. Some of these implanted BMDEPCs were stained as CD31+ (Fig. 7) and VWF+ (Fig. 8) cells.

*Quantitative Analysis of IF and Immunohistochemical Staining of Ischemic Quadriceps on Day 21 After CLI Procedure (Figs. 7, 8, and 9).* The results of IF staining showed a remarkably lower number of CD31+ (Fig. 7) and vWF+ (Fig. 8) cells, indexes of endothelial cells/angiogenesis, in group 2 animals than in other groups. Similarly, the number was lower in groups 1, 3, and 4 than in group 5, but did not differ among groups 1, 3, and 4. Furthermore, the results of immunohistochemical staining demonstrated a remarkably lower number of small vessels (defined as <15 μm) in group 2 than in other groups (Fig. 9). It was also notably lower in groups 1, 3, and 4 than in group 5, but did not differ among groups 1, 3, and 4. Both IF and immunohistochemical findings (Figs. 7, 8, and 9) imply that combined therapy with BMDEPCs and ECSW is better than either one alone in enhancing angiogenesis in the ischemic muscle.

## DISCUSSION

*Principal Findings of the Current Study.* A variety of animal models (11–13, 23) and some clinical trials (24, 25) have shown that EPC therapy is effective in improving limb ischemia. In the current study, we found that, compared with the CLI control group, the ratio of INBF was remarkably improved in the BMDEPC therapy group. The results of the current study, therefore, reinforce the findings of the previous studies (11–13, 23–25).

Interestingly, while improvement of hind limb ischemia after EPC therapy has been reported in previous studies (11–13,
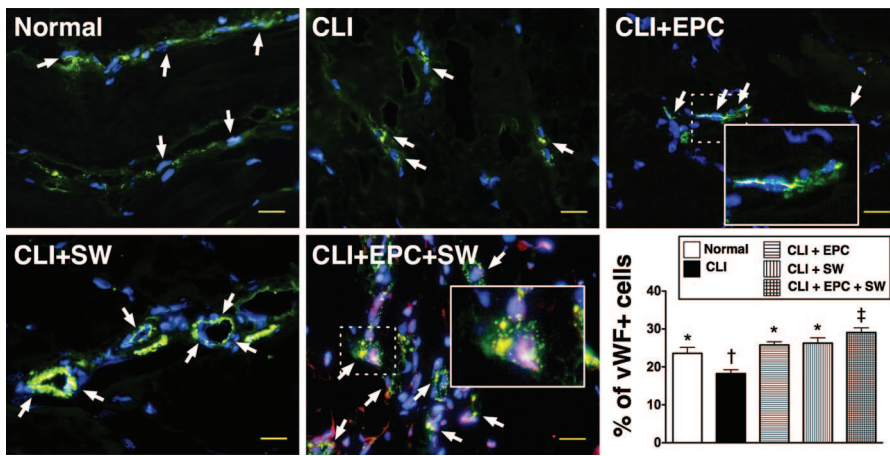
Figure 8. Immunofluorescence microscope (×400) findings of von Willebrand factor (*vWF*)+ stained cells, an indicator of endothelial cells, in ischemic area of each group (n = 6) on day 21 following critical limb ischemia (*CLI*) procedure. The number of vWF+ cells (*white arrows*) (identification by blue color [DAPI for nuclei] and green color [vWF+ staining]) was remarkably lower in the CLI group than in other groups. Conversely, the number of vWF+ cells was substantially higher in the CLI + endothelial progenitor cell (*EPC*) + shock wave (*SW*) group than in other groups. The *square box* shows the double stain of vWF+ cells (Dil dye and vWF+ staining) under high magnification of the *dotted box* in the CLI + EPC and CLI + EPC + SW groups, indicating these cells were derived from implanted EPCs after 21-day cell culture. Symbols (*, †, ‡) indicate significant difference (at .05 level) by Tukey multiple comparison procedure. The scale bars in right lower corner represent 20 μm.
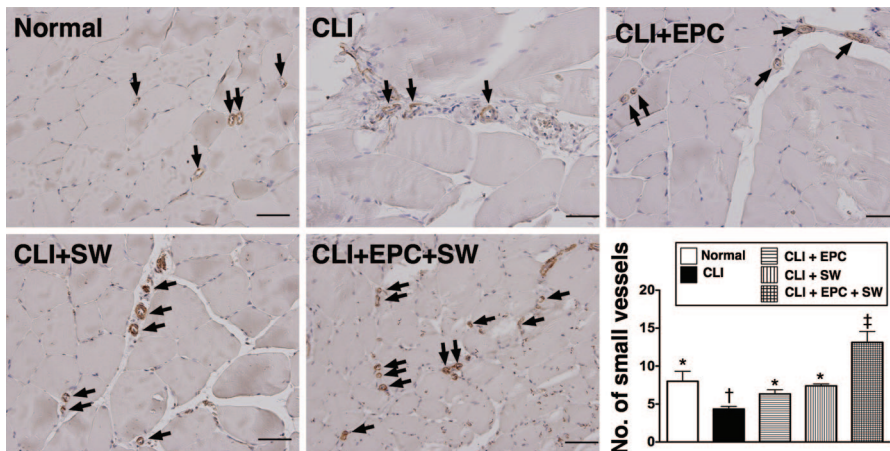


Figure 9. Number of vessels quantification (×200) in ischemic area for each group (n = 6) on day 21 following critical limb ischemia (*CLI*) induction. α-smooth muscle actin immunohistochemical staining showing a notably higher number of small vessels (*black arrows*) (≤15 μm in diameter) in the CLI + endothelial progenitor cell (*EPC*) + shock wave (*SW*) group than in other groups, whereas the number of small vessels was notably lower in the CLI group than in normal, CLI + EPC, and CLI + SW groups. Scale bars in right lower corner represent 50 μm. Symbols (*, †, ‡) indicate significant difference (at .05 level) by Tukey multiple comparison procedure.

19, 23–25), the impact of ECSW therapy on the outcome of CLI has seldom been investigated (18). One essential finding in the current study is that ECSW therapy was not inferior to BMDEPC administration in improving the ratio of INBF in the animals. Furthermore, the principal finding is that combined therapy with BMDEPCs and ECSW was superior to either therapy alone in increasing the ratio of INBF in this experimental setting. The results of the present study, therefore,

extended the findings of previous reports (11–13, 18, 23–25). The most important finding in the present study is the fact that this combined treatment strategy is ready for clinical application.

*The Mechanisms Underlying Improvement in CLI Using Combined Therapy With BMDEPCs and ECSW.* The improvement in blood flow after EPC therapy in the setting of CLI has been reported to be mainly due to the incorporation of implanted stem cells into the growing col-

lateral vasculature (19) and their participation in angiogenesis (11, 13). In concert with this proposal, the current study demonstrated notable enhancement in both mRNA and protein expressions of endothelial nitric oxide synthase in animals with CLI after BMDEPC therapy. In addition, the protein expressions of VEGF, SDF-1α, and chemokine receptor type 4 were substantially upregulated in animals after BMDEPC administration. Furthermore, a drastic increase was noted in the numbers of CD31+ and vWF+ cells in the hind limb ischemic area on IF staining after BMDEPC implantation. These findings, in addition to supporting those of previous studies (19), could explain the remarkable elevations in both the vessel density in the ischemic area and the ratio of INBF in animals with BMDEPC treatment compared with those without.

*In vitro* and *in vivo* studies (14, 15, 26) have demonstrated that not only can SW upregulate VEGF, but it can also enhance EPC proliferation and recruit EPCs to ischemic areas for angiogenesis (18). However, the principal mechanism underlying improved blood flow in CLI following ECSW therapy has not been fully investigated (18). Interestingly, compared with BMDEPC treatment, ECSW therapy offered similar therapeutic advantage in the upregulation of these angiogenic/endothelial biomarkers and improving the ratio of INBF in animals with CLI. In this way, our findings are supported by the results of previous studies (14, 15, 26). Furthermore, the results of the present study support that ECSW therapy enhanced numbers of CD31+ and vWF+ cells (Figs. 7 and 8) in the ischemic area through enhancing local production of SDF-1α. Importantly, the current study demonstrated that combined therapy with BMDEPCs and ECSW was superior to either treatment option alone in enhancing the expressions of angiogenic/endothelial biomarkers, enriching the numbers of EPCs (i.e., CD31+ and vWF+ cells) in the ischemic area, and increasing blood flow in the hind limb ischemic region. Thus, we suggest that the enhanced accumulation of EPCs in the ischemic area after ECSW therapy may be due to both an increased SDF-1α level for EPC homing and the retaining effect of this chemokine on implanted EPCs. Therefore, this could, at least in part, explain the augmented angiogenesis through combined therapy with EPCs and ECSW. Accordingly, our findings, in addition to reinforcing the proposal of
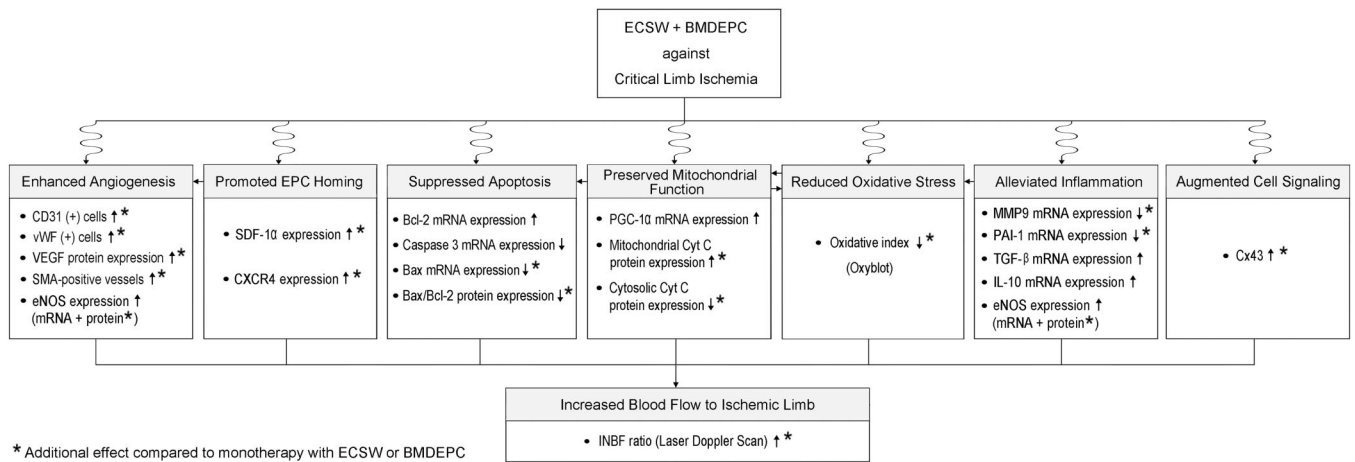
**Figure 10.** Proposed mechanisms underlying therapeutic effects of endothelial progenitor cell (*EPC*), shock wave (*SW*), and combined EPCs and SW on improving critical limb ischemia. *ECSW*, extracorporeal shock wave; *BMDEPCs*, bone marrow-derived endothelial progenitor cells; *vWF*, von Willebrand factor; *VEGF*, vascular endothelial growth factor; *SMA*, α-smooth muscle actin; *eNOS*, endothelial nitric oxide synthase; *SDF-1á*, stromal cell-derived factor-1α; *CXCR4*, chemokine receptor 4; *Cyt C*, cytochrome c; *PGC-1á*, peroxisome proliferator-activated receptor-γ coactivator-1α; *MMP9*, matrix metalloproteinase 9; *PAI-1*, plasminogen activator inhibitor-1; *TGF-â*, transforming growth factor-β; *IL-10*, interleukin-10; *Cx43*, connexin 43; *INBF*, ischemic/normal blood flow.

ECSW-elicited improvement in hind-limb perfusion in the setting of CLI, mainly through angiogenesis/vasculogenesis, raise the concern of potential clinical application of a combined BMDEPC and ECSW treatment in patients with CLI.

*Additional Benefit Other Than Angiogenesis in Improving CLI Following BMDEPCs and ECSW Therapy.* Interestingly, ECSW has been successfully utilized in treating shoulder tendinitis and plantar fasciitis (16, 17). At a biological level, previous molecular/cellular studies (20, 26, 27) have demonstrated that ECSW therapy attenuates polymorphonuclear neutrophil and macrophage infiltration as well as expression of acute proinflammatory cytokines over the wound in animal models. On the other hand, stem cell therapy has been found to possess a distinctive immunomodulatory property that contributes to the downregulation of inflammatory reaction in ischemic conditions (22, 28, 29). In the current study, we showed that the gene expressions of matrix metalloproteinase-9 and plasminogen activator inhibitor-1, as well as the oxidative stress, were remarkably attenuated, whereas the gene expression of interleukin-10 was notably increased in animals treated by BMDEPCs or ECSW. Therefore, our findings are consistent with those of previous studies (22, 28, 29). In addition, both gene and protein expressions of apoptotic biomarkers were substantially downregulated, whereas those of antiapoptosis were markedly upregulated following either BMDEPCs or ECSW treatment. This

antiapoptotic action was found to be more pronounced in the combined treatment group. These findings and those of enhanced angiogenesis may explain the preservation of mitochondrial cytochrome c, proliferator-activated receptor-γ coactivator-1α, and connexin 43, an index of cellular signal transduction between skeletal muscle cells, in animals after combined therapy. Taken together, our findings showed that combined therapy with BMDEPCs and ECSW was superior to either treatment strategy alone in regulating these biomarkers in the setting of CLI.

The precise mechanism underlying combined EPCs and ECSW treatment for CLI may be more complex. The proposed mechanisms of the potential therapeutic impact of combined therapy with EPCs and ECSW on CLI have been summarized in Figure 10.

In conclusion, a combination of autologous BMDEPC and ECSW offered additional benefit in restoring hind-limb perfusion compared with either treatment option alone in the setting of rodent CLI.

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