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Extracorporeal shock wave-assisted adipose-derived fresh stromal vascular fraction restores the blood flow of critical limb ischemia in rat

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ABSTRACT

We tested the hypothesis that extracorporeal-shock-wave (ECSW)-assisted adipose-derived stromal vascular fraction (SVF) therapy was better than either one for restoring the blood flow in critical limb ischemia (CLI). Adult male-SD rats were categorized into group 1 (sham-operated-control), group 2 (CLI), group 3 [CLI + ECSW (280 impulses/0.10 mJ/mm^2) applied to left inguinal area at 3 h after CLI], group 4 [CLI + SVF (1.2×10^6) implanted into CLI area at 3 h after CLI], group 5 (CLI + ECSW-SVF). In vitro studies showed that ECSW significantly enhanced angiogenesis in human umbilical-vein endothelial cells and carotid-artery ring, and SVF significantly suppressed inflammation (TNF-α/NF-Kb/IL-1ß/MMP-9) in smooth-muscle cells treated by LPS (all $p < .001$). By day 14 after CLI, the ratio of ischemic/normal blood flow (INBF) was highest in group 1, lowest in group 2, significantly higher in group 5 than in groups 3 and 4, but no difference was shown between the latter two groups (all $p < .001$). The fibrotic area in CLI region exhibited an opposite pattern of INBF ratio (all $p < .0001$). Protein (CD31/vWF/eNOS) and cellular (CD31/vWF) expressions and number of small vessels in CLI area exhibited an identical pattern, whilst protein expressions of apoptotic (caspase3/PARP/mitochondrial-Bax) fibrotic/DNA-damaged (Samd3/TFG-ß/γ-H2AX) biomarkers exhibited an opposite pattern to INBF among five groups (all $p < .0001$). The numbers of angiogenetic cells in CLI region (SDF-1α/VEGF/CXCR4) and endothelial-progenitor cells (C-kit/CD31+//Sca-1/CD31+//CD34/KDR+/VE-cadherin/CD34+) in circulation significantly and progressively increased from groups 2 to 5 (all $p < .0001$). In conclusion, ECSW-SVF therapy effectively enhanced angiogenesis and restoration of blood flow in CLI area.

1. Introduction

Cardiovascular disease (CVD) remains the leading cause of death worldwide [1]. The CVD can be categorized into heart disease and peripheral vascular occlusive disease (PAOD) [2]. In fact, PAOD, a typical atherosclerotic disease, is one of the major contributors for CVD [2,3]. Epidemiologic and clinical observation studies [4–6] have established that up to 10% of population younger than 70 years, 15% to 20% between 70 and 85 years and 50% in those > 85 years have PAOD, highlighting that PAOD is common in older people. In our clinical practice, the PAOD which is further subdivided into symptomatic and asymptomatic types has an estimated prevalence of up to 13% in age group over 50 years old [4]. Additionally, about 35% symptomatic patients will eventually develop into intermittent claudication (IC) or

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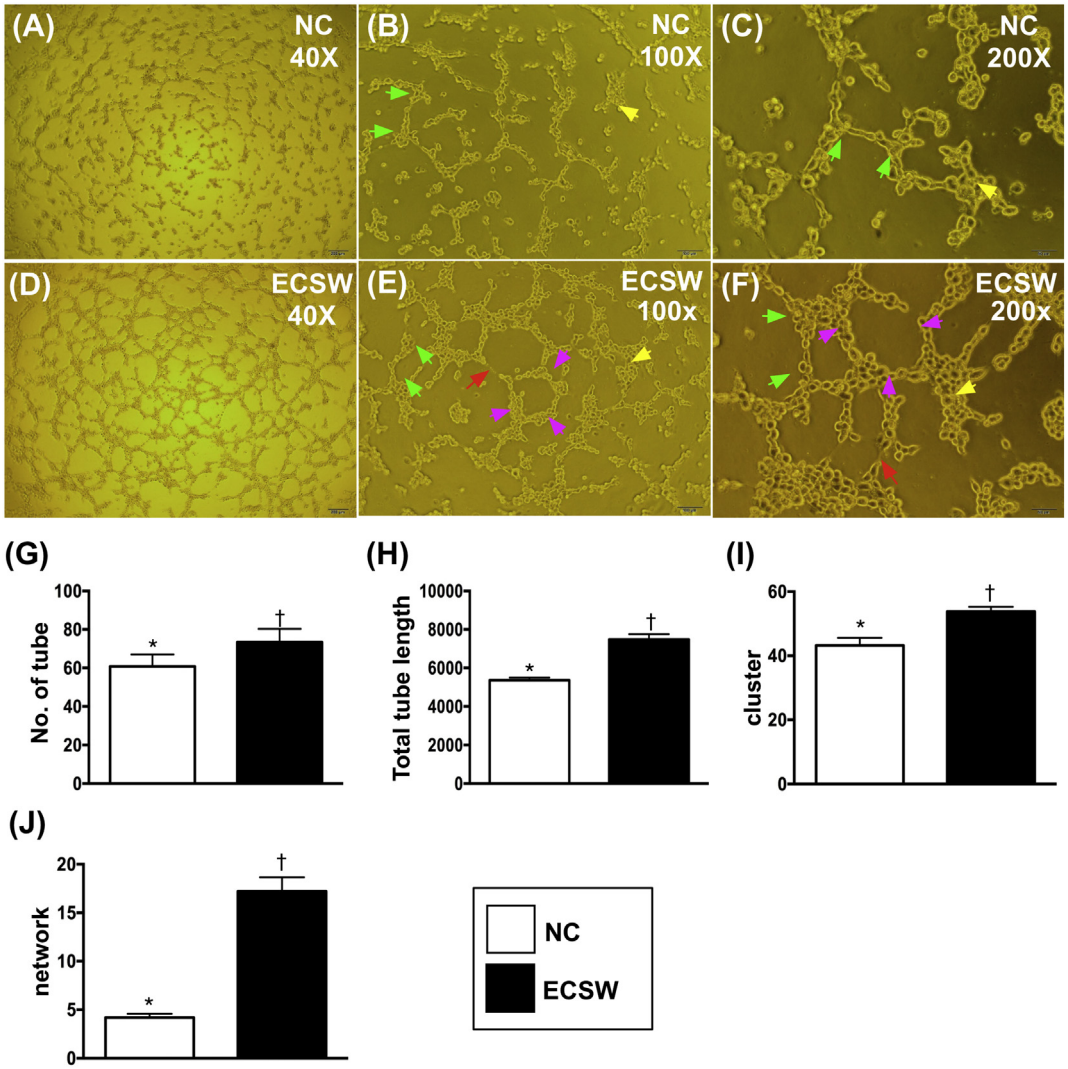

Fig. 1. ECSW treatment augmented angiogenesis of HUVECs.
A to F) Illustrating the microscopic findings (40×, 100×, 200×) of Matrigel assay for identification of tubular length (green arrows), tubular formation (red arrows), cluster formation (yellow arrows), and network formation (pink arrows). **G)** Number of tubules, * vs. †, $p < .05$. **H)** Tubular length, * vs. †, $p < .01$. **I)** Cluster formation, * vs. †, $p < .01$. **J)** Network formation, * vs. †, $p < .0001$. HUVECs = human umbilical vein endothelial cells; NC = normal control; ECSW = extracorporeal shock wave. $n = 6$ in each group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

critical limb ischemia (CLI) [7].

PAOD, a well-known progressive atherosclerotic disorder, has become a global problem [8] that can lead to poor quality of life [9], an increased risk of hospitalization and limb amputation [10], along with unacceptable high costs of care [11,12]. Of importance is that PAOD is not only an independent predictor of CVD mortality but also causes unfavorable high long-term morbidity and mortality [13–16] regardless of recent advances in PAOD treatment [16], especially in those of CLI [10] and diabetic patients [17,18].

Treatment for CLI/PAOD still poses as a formidable challenge to clinicians [19]. Percutaneous transluminal angioplasty (PTA) or peripheral bypass graft are currently two common methods for CLI [12,13,16]. However, requirement of repeated PTA, secondary bypass surgery, or eventually amputation due to recurrent CLI are the limitations of these interventional procedures [13]. These could explain why the long-term outcome remains regrettably favorable in CLI/PAOD patients [20–22]. In view of lacking an effective treatment for CLI/PAOD, developing a new strategic management of this disease entity is urgent and fundamentally important.

Growing data has shown that treatment with adipose-derived fresh stromal vascular fraction (SVF) containing primitive stem cells, which could be utilized immediately after isolation without need for further cell culture, speeds up wound healing via angiogenesis, growth factor production and anti-inflammation [23]. Basic research has further identified that adipose-derived fresh SVF-enriched heterogeneous populations of undifferentiated, mononucleated elements, as based on cell surface antigens within those multipotent tissues [23,24], are emerging as an easy and safe way to treat various diseases [23–27]. However, the therapeutic potential of SVF on restoration of blood flow in CLI area has seldom been reported [28].

Extracorporeal shock wave (ECSW) therapy has been established to effectively improve ischemia-related organ dysfunction mainly through enhancing angiogenesis, up-regulating SDF-1α expression, recruiting endothelial progenitor cells, and by suppressing inflammation [29–31]. We have further established that combined therapy with ECSW and bone marrow-derived mesenchymal stem cells was superior to either one alone for improving LVEF, reducing infarct size, and inhibiting LV remodeling [30]. Accordingly, we tested the hypothesis that combined ECSW-SVF could be superior to either one alone for restoring the blood flow in ischemic area in a rodent CLI model.

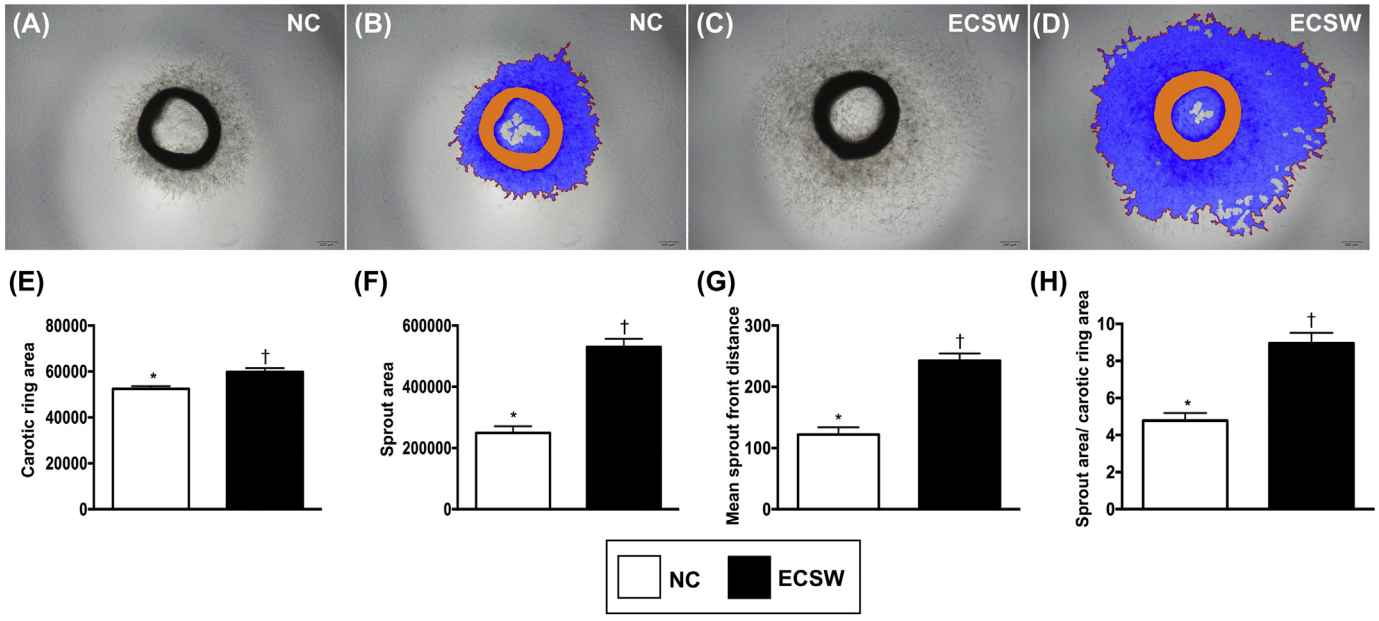

Fig. 2. ECSW treatment enhanced angiogenesis in rat carotid artery ring culture.
A to D) Illustrating the photographic imaging (100×) of angiogenesis from rat carotid artery ring culture. **E)** Analytical result of carotid ring area, * vs. †, p < .01. **F).**
Analytical result of sprout area, * vs. †, $p < .0001$. **G)** Analytic result of mean sprout front distance, * vs. †, $p < .001$. **H)** Analytic result of ratio of sprout area to carotid ring area, * vs. †, $p < .0001$. NC = normal control; ECSW = extracorporeal shock wave. n = 6 in each group.

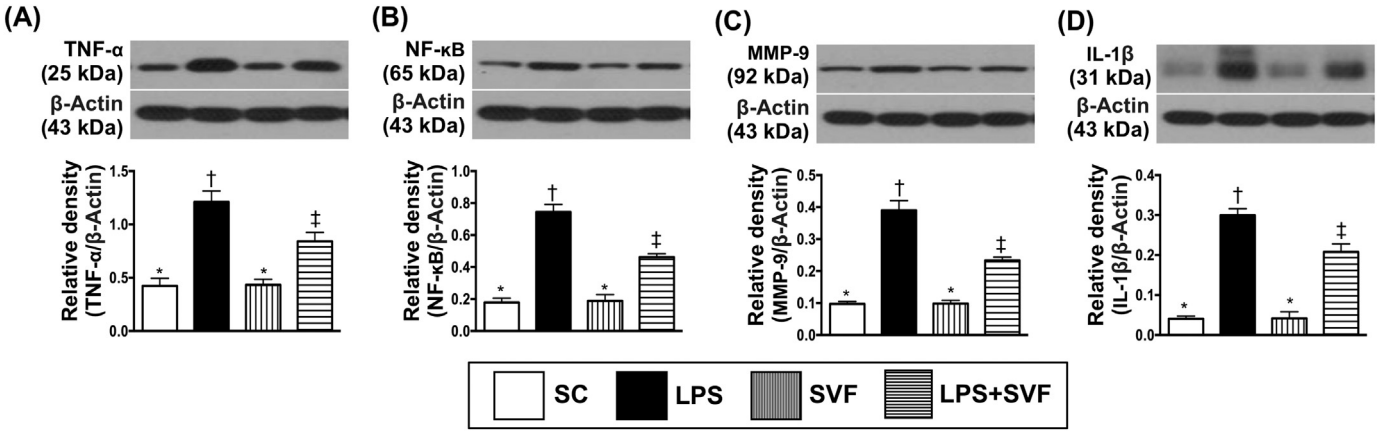

Fig. 3. SVF treatment suppressed the inflammatory reaction in A7r5 cell line.
A) Protein expression of tumor necrosis factor (TNF)-1α, * vs. other groups with different symbols (†, ‡), p < .001. **B)** Protein expression of nuclear factor (NF)-κB, * vs. other groups with different symbols (†, ‡), $p < .001$. **C)** Protein expression of matrix metalloproteinase (MMP)-9, * vs. other groups with different symbols (†, ‡), p < .001. **D)** Protein expression of interleukin (IL)-1ß, * vs. other groups with different symbols (†, ‡), p < .001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 4$ for each group). Symbols (*, †, ‡) indicate significance (at 0.05 level). NC = normal control; ECSW = extracorporeal shock wave; LPS = lipopolysaccharide; SVF = stromal vascular fraction.

2. Materials and methods

2.1. Ethics

All animal procedures were approved by the Institute of Animal Care and Use Committee at Kaohsiung Chang Gung Memorial Hospital (Affidavit of Approval of Animal Use Protocol No. 2016012703) and performed in accordance with the Guide for the Care and Use of Laboratory Animals.

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

2.2. The procedure and protocol of critical limb ischemia (CLI)

The procedure and protocol have been described in our previous reports in details [31,32]. In brief, pathogen-free, adult male Sprague Dawley (SD) rats weighing 325–350 g (Charles River Technology, Bio-LASCO, Taiwan) in CLI group 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. 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 controls, the arteries were only isolated without ligation.

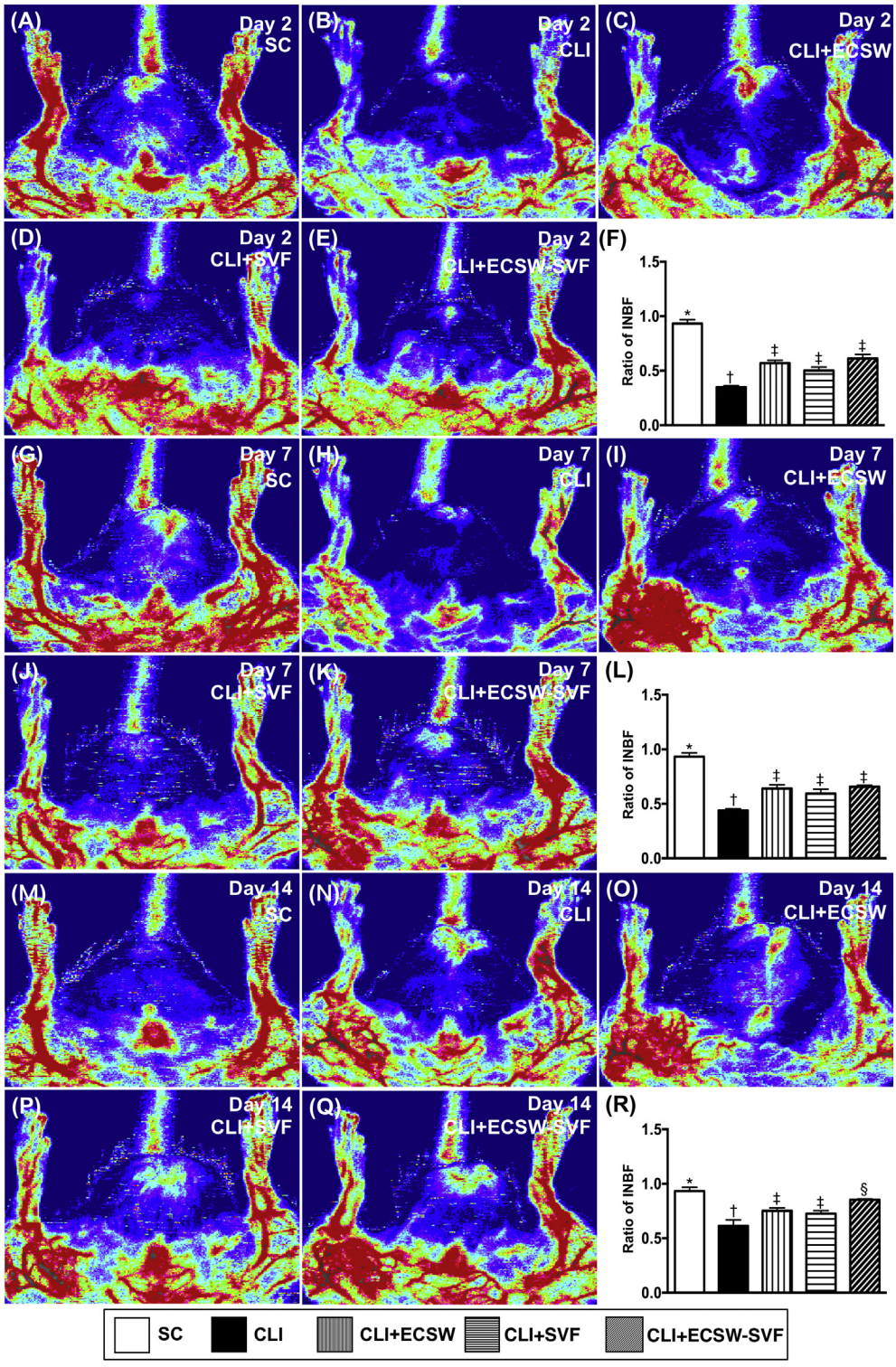

Fig. 4. Time courses of blood flow in CLI area.
A to E) Illustrating the Laser doppler finding for identification blood flow at both limbs in each group by day 2 after CLI procedure. **F)** Analytical result of ratio of ischemic/normal blood flow (INBF), * vs. other groups with different symbols (†, ‡), $p < .0001$. **G to K)** Illustrating the Laser doppler finding for identification blood flow at both limbs in each group by day 7 after CLI procedure. **L)** Analytical result of INBF ratio at day 7, * vs. other groups with different symbols (†, ‡), $p < .0001$. **M to Q)** Illustrating the Laser doppler finding for identification blood flow at both limbs in each group by day 14 after CLI procedure. **R)** Analytical result of INBF ratio at day 14, * vs. other groups with different symbols (†, ‡, §), p < .0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 8$ for each group). Symbols (*, †, ‡, §) indicate significance (at 0.05 level). CLI = critical limb ischemia; SC = sham control; ECSW = extracorporeal shock wave; SVF = stromal vascular fraction.

2.3. Animal grouping and strategic treatment

Forty SD rats were categorized into five groups: sham-operated control (SC) (group 1), CLI (only treated with culture medium 1.0 cc over the femoral area) (group 2), CLI + ECSW (280 impulses at 0.10 mJ/mm^2, applied to left inguinal area at 3 h after CLI) (group 3), CLI + autologous SVF [(1.2×10^6) implanted into CLI area at 3 h after CLI procedure] (group 4) and CLI + ECSW + autologous SVF (group 5).

The ECSW machine was applied in the present study was Storz Duolith SD1 (STORZ MEDICAL AG; Switzerland). Additionally, the energy dosage of ECSW (280 impulses at 0.1 mJ/mm^2) utilized in this study was based on our recent reports with minimal modification [31,33].

2.4. Procedure and protocol for isolation of adipose-derived fresh SVF

The procedure and protocol for preparing autologous SVF (i.e., the isolated SVF only for the same animal used) were based on the previous report [34]. In detail, animals in CLI + SVF and CLI + ECSW-SVF were

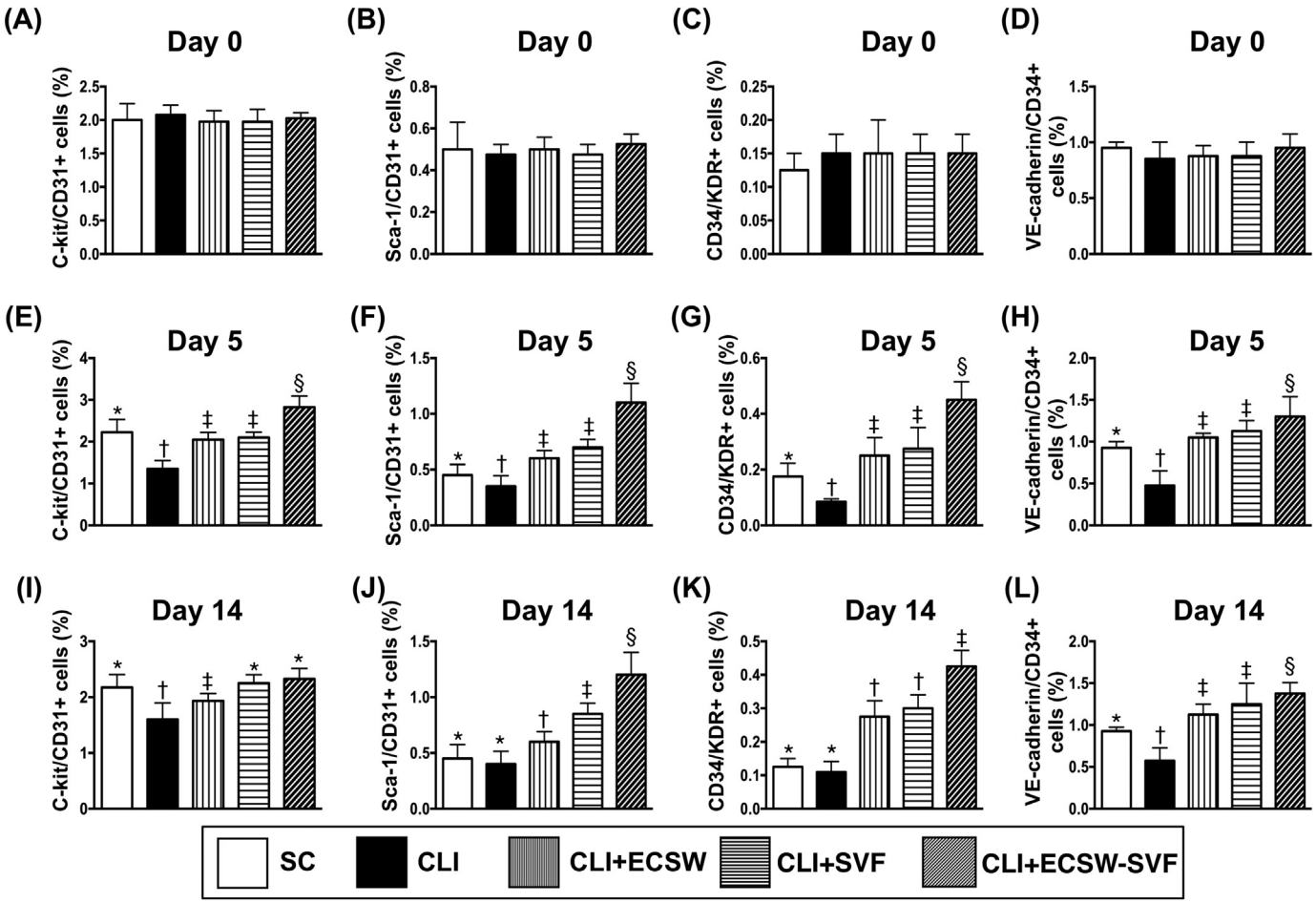

Fig. 5. Time courses of circulating levels of endothelial progenitor cells.
A to D) By day 0, analytical result of the circulating numbers of C-kit/CD31 +, Sca-1/CD31 +, CD34/KDR + and VE-cadherin/CD34 + cells, respectively, all $p > .5$. **E)** By day 5, analytical result of number of C-kit/CD31 + cells, * vs. other groups with different symbols (†, ‡, §), $p < .0001$. **F)** By day 5, analytical result of number of Sca-1/CD31 + cells, * vs. other groups with different symbols (†, ‡, §), $p < .0001$. **G)** By day 5, analytical result of number of CD34/KDR + cells, * vs. other groups with different symbols (†, ‡, §), $p < .0001$. **H)** By day 5, analytical result of number of VE-cadherin/CD34 + cells, * vs. other groups with different symbols (†, ‡, §), $p < .0001$. **I)** By day 14, analytical result of number of C-kit/CD31 + cells, * vs. other groups with different symbols (†, ‡), $p < .001$. **J)** By day 14, analytical result of number of Sca-1/CD31 + cells, * vs. other groups with different symbols (†, ‡, §), $p < .0001$. **K)** By day 14, analytical result of number of CD34/KDR + cells, * vs. other groups with different symbols (†, ‡), $p < .0001$. **L)** By day 14, analytical result of number of VE-cadherin/CD34 + cells, * vs. other groups with different symbols (†, ‡, §), $p < .0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 8 for each group). Symbols (*, †, ‡, §) indicate significance (at 0.05 level). CLI = critical limb ischemia; SC = sham control; ECSW = extracorporeal shock wave; SVF = stromal vascular fraction.

anesthetized with inhalational isoflurane 3 h prior to CLI induction. Both inguinal regions were clipped and prepared with 10% povidone iodine, the inguinal fat pads were removed, and a 1 × 1 × 1 cm (1 cm^3) of adipose tissue was excised from each. The incisions were closed with 3/0 silk sutures. We pooled all cells from the same rat to make a master batch of SVF. The excised adipose tissues were washed extensively by phosphate buffer solution (PBS, Sigma1) to remove contaminating debris and red blood cells, and then minced with fine tissue scissors. The fragmented tissues were incubated with 0.1% collagenase (collagenase from Clostridium histolyticum C0130, Sigma1) and kept in a slow shaking water bath at 37 °C for 60 min. Collagenase was then removed by diluting the samples with PBS. The cell suspension was centrifuged twice at 1300 rpm (260G) for 5 min. The supernatant containing mature adipocytes was removed. The precipitate was passed through 100 μm mesh filter and used as SVF. Viable cells were counted (Thoma slide) by adding trypan blue to SVF. There were approximately 4 × 10^6/ml viable cells.

2.5. Measurement of blood flow with laser Doppler

The procedure and protocol were based on our previous reports [31,32]. 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 euthanized. 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 to the right (normal) leg was computed. By day 14, the rats were euthanized and the quadriceps muscle was collected for individual study.

2.6. Assessment of rat aortic-ring angiogenesis

To test the therapeutic capacity of ECSW on exo vivo angiogenesis, assessment of rat aortic-ring angiogenesis was performed. The procedure and protocol of ECSW on exo vivo angiogenesis have been described in our recent report [35]. In brief, rat carotid ring angiogenesis was assessed in 24-well tissue culture plates embedded with 150 μL of 1 mg/mL type I collagen (BD Biosciences, Franklin Lakes, NJ, USA) and

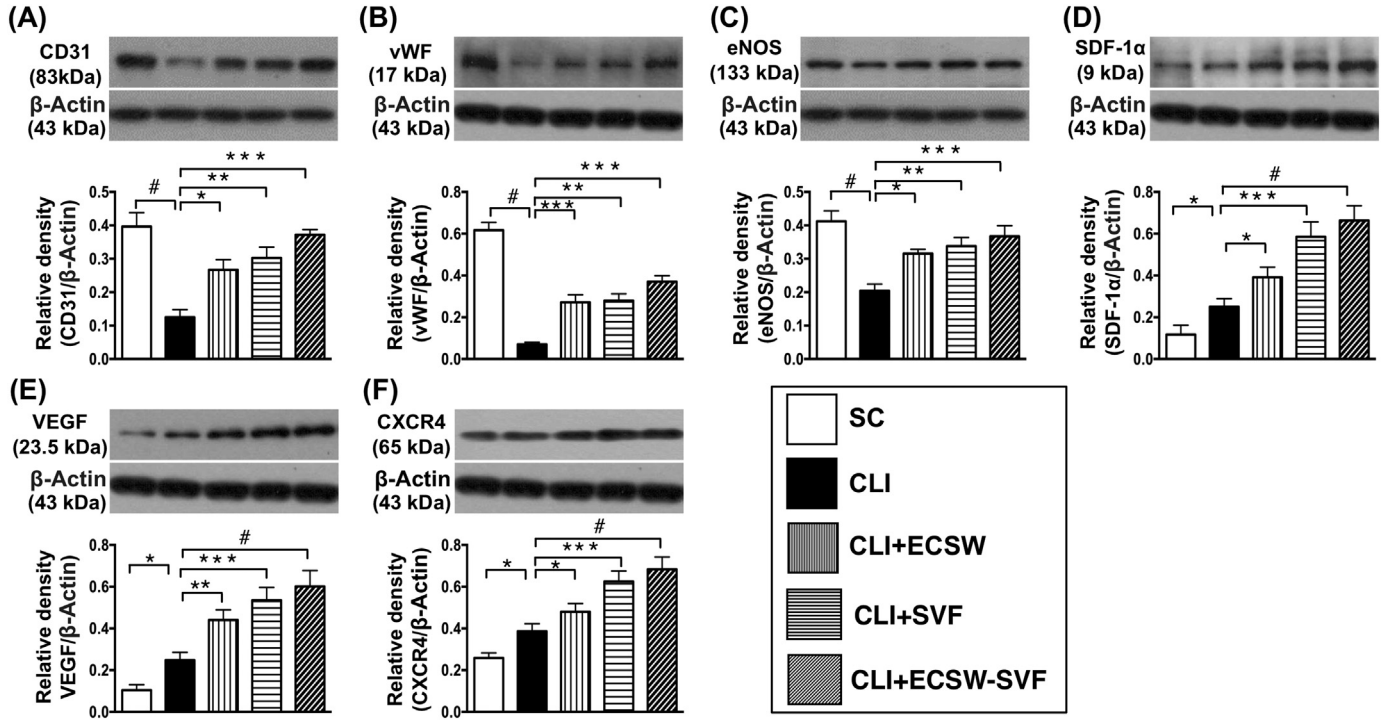

Fig. 6. The protein expressions of endothelial and angiogenesis biomarkers in ischemic quadriceps muscle by day 14 after CLI procedure.
A to F) Representing the protein expressions of CD31 (A), von Willebrand factor (vWF) (B), endothelial nitric oxide synthase (eNOS) (C), stromal cell-derived factor (SDF)-1α (D), vascular endothelial growth factor (VEGF) (E) and CXCR4 (F), ** indicated $p < .01$; *** indicated $p < .001$; # indicated $p < .0001$. CLI = critical limb ischemia; SC = sham control; ECSW = extracorporeal shock wave; SVF = stromal vascular fraction. $n = 6$ in each group.

allowed to gel for 60 min at 37 °C and 5% CO2. The carotid arteries were excised from 4 rats 3 h after receiving ECSW therapy (120 impulses at 0.10 mJ/mm^2), followed by removal of all extraneous tissue with forceps and a scalpel. On the other hand, another 4 carotid arteries from additional 4 rats without ECSW treatment were also harvested and served as control group. The carotid artery was then cut into 1 mm cross-section pieces, placed in collagen-coated wells before filling with 500 μL of serum-free MCDB131 medium. The carotid rings were incubated for 12 h at 37 °C and 5% CO2. Photographs were taken at day 12 with 12.5 times magnification. The number and length of sprouting vessels were quantified by ULYMPUS DP72 software. Experiments were performed twice.

2.7. Matrigel assay for evaluating angiogenesis

To elucidate the therapeutic effect of ECSW on in vitro angiogenesis, Matrigel assay was performed in the present study. The protocol and procedure of assessment of angiogenesis were based on our recent report [35] with some modifications. In brief, human umbilical vein derived endothelial cells (HUVECs) were purchased from BCRC (Bioresource Collection and Research Center, Taiwan) and placed in 48-well plates at 3.0×10^4 cells/well in 100 μL serum-free M199 culture medium mixed with 100 μL cold Matrigel (Chemicon International, Inc., Temecula, CA, USA) for 3 h and incubated at 37 °C in 5% CO2. Three random microscopic images (200×) were taken from each well to count cluster, tube and network formations, and the mean values were obtained. Both cumulative and mean tube lengths were calculated by Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA).

2.8. Utilization of A7r5 smooth muscle cells for determining the capacity of SVF on suppressing the inflammatory reaction

To assess the impact of SVF treatment on suppressing inflammation, the A7r5 smooth muscle cell line (Bioresource Collection and Research Center, Taiwan) was utilized in the in vitro study which was designed as four groups: (1) A7r5 (4.0×10^4 cells), (2) A7r5 + lipopolysaccharide (LPS) (1.0 μM), (3) A7r5 + SVF (5.0×10^5 cells) and (3) A7r5 + SVF cultured for 3 h, then added LPS co-cultured for another 3 h.

2.9. Flow cytometric quantification of numbers of circulating endothelial progenitor cells (EPCs)

Flow cytometric analysis for identification of EPC surface markers was performed based on our previous reports [19]. Briefly, the cells were immunostained for 30 min with PE-conjugated antibodies against CD31 (BioLegend, San Diego, CA, USA), KDR (BD Pharmingen, San Jose, CA, USA), CD34 (BD Pharmingen) and FITC-conjugated antibodies against Sca-1 (BD Pharmingen), c-Kit (BD Pharmingen), CXCR4 (BD Pharmingen). Isotype-identical antibodies (IgG) served as controls. Flow cytometric analyses were performed by utilizing a fluorescence-activated cell sorter (Beckman Coulter FC500 flow cytometer; Indianapolis, IN, CA).

2.10. Immunofluorescent (IF) staining

The IF stain was performed for the assessment of CD31 + (1:100, Bio-Rad), von Willebrand factor (vWF) + (1:200, Merck Millipore), CXCR4 + (1:200, Bioss), vascular endothelial growth factor (VEGF) (1:400, Abcam), γ-H2AX + (1:500, Abcam), matrix metalloproteinase (MMP)-9 + (1:200, Invitrogen) and F4/80 + (1:100, Santa Cruz) cells ($n = 6$ for each group) using respective primary antibodies based on our recent study [31]. Irrelevant antibodies were used as controls in the current study.

2.11. Western blot analysis

The procedure and protocol of western blot analysis were based on our previous reports [35,36]. In brief, equal amounts (30 μg) of protein

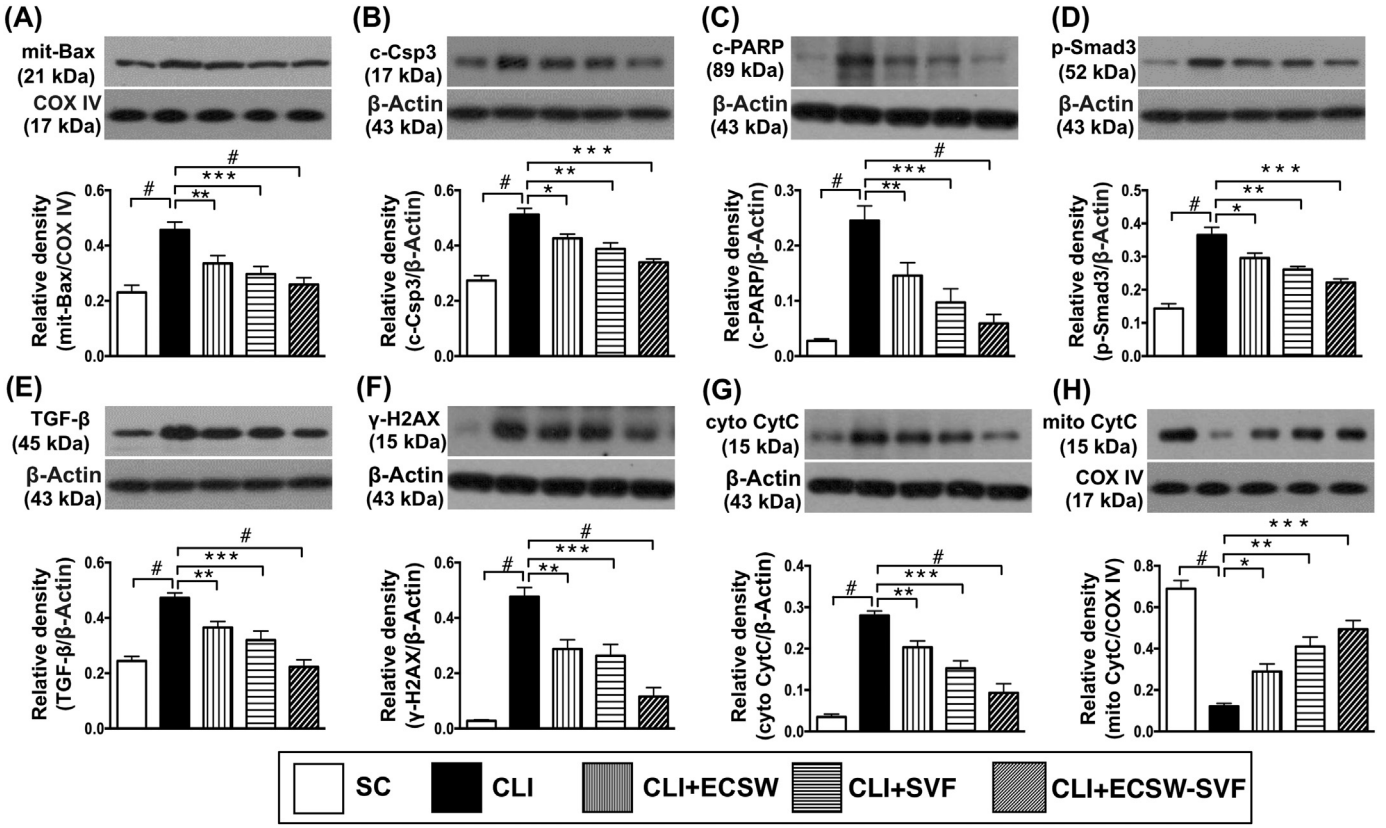

Fig. 7. Protein expressions of apoptotic, fibrotic and mitochondrial DNA-damaged biomarkers in ischemic quadriceps muscle by day 14 after CLI procedure.
A to H) Representing the protein expressions of mitochondrial Bax (mito-Bax) (A), cleaved caspase 3 (c-Casp3) (B), C-PARP (C), Smad3 (D), transforming growth factor (TGF-ß) (E), γ-H2AX (F), cytosolic cytochrome C (cyt-cyto C) (G), and mitochondrial cytochrome C (mit-cyto C) (H). * indicated $p < .05$; ** indicated $p < .01$; *** indicated $p < .001$; # indicated $p < .0001$. CLI = critical limb ischemia; SC = sham control; ECSW = extracorporeal shock wave; SVF = stromal vascular fraction. $n = 6$ in each group.

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 CXCR4 (1: 1000, Abcam), vascular endothelial growth factor (VEGF) (1: 1000, Abcam), stromal cell-derived growth factor (SDF)-1α (1: 1000, Cell Signaling), von Willebrand factor (vWF) (1: 1000, Abcam), CD31 (1: 1000, Abcam), cytosolic cytochrom C (1: 1000, BD), mitochondrial cytochrome C (1: 1000, BD), endothelial nitric oxide synthase (eNOS) (1: 1000, Abcam), mitochondrial Bax (1: 1000, Abcam), cleaved caspase 3, cleaved poly ADP ribose polymerase (PARP) (1:1000, Cell Signaling), γ-H2AX (1:1000, Cell Signaling), phosphorylated (p)-Smad3 (1:1000, Cell Signaling), transforming growth factor (TGF)-β (1: 5000, Abcam), tumor necrosis factor (TNF)-α (1:1000, Cell Signaling), nuclear factor (NF)-κB (1: 1000, Abcam), matrix metalloproteinase (MMP)-9 (1: 1000, Abcam), interleukin (IL)-1β (1:1000, Cell Signaling) and actin (1: 10000, Chemicon) for 1 h at room temperature. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (1:2000, Cell Signaling) was used as a secondary antibody for one-hour incubation at room temperature. The washing procedure was repeated eight times within 1 h. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposed to Biomax L film (Kodak). For quantification, ECL signals were digitized using Labwork software (UVP).

2.12. Measurement of small vessels in CLI region

The procedure and protocol have been described in our previous reports [32,35]. In detail, immunohistochemical (IHC) staining of blood vessels was performed 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 g/tablet) (Sigma) was added, followed by washing with PBS three times after 1 min. Finally, hematoxylin was added as a counter-stain 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 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.

2.13. Statistical analysis

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

3. Results

3.1. ECSW treatment augmented angiogenesis of HUVECs and carotid ring (Figs. 1 and 2)

As compared with control group, ECSW therapy significantly enhanced angiogenesis of HUVECs, including number of tubule, and cluster and network formations (Fig. 1).

To investigate the therapeutic effect of ECSW treatment on functional integrity of arteries, rat carotid ring culture was conducted. Consistently, the results demonstrated an identical pattern between

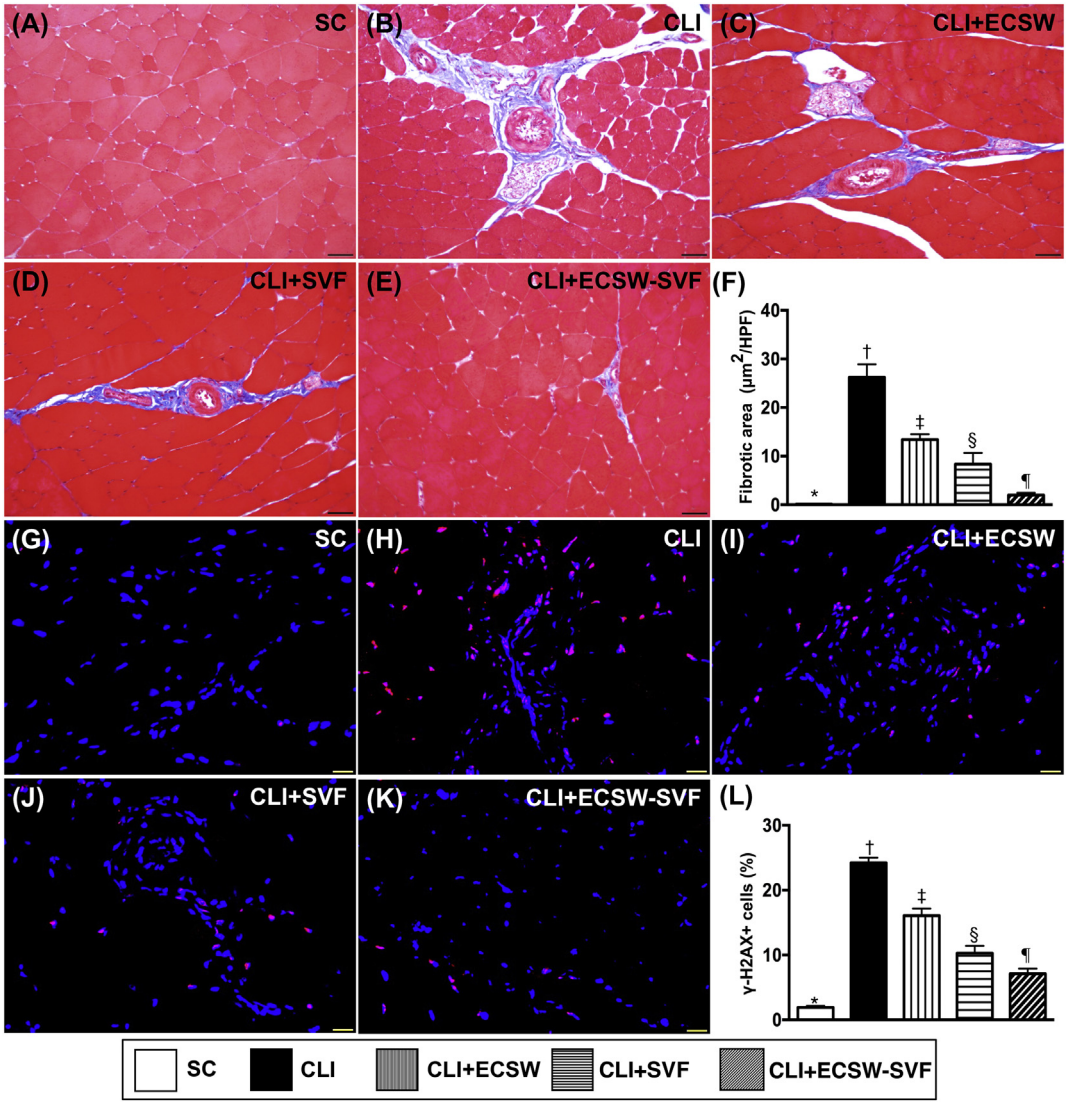

Fig. 8. Histopathological findings of fibrotic area and DNA-damaged cell expression in ischemic quadriceps muscle by day 14 after CLI procedure.
A to E) Illustrating the immunohistochemical microscopic finding (200×) of Masson's trichrome stain for identification of fibrotic area (blue color) in CLI quadriceps muscle. Scale bars in lower right corner represent 50 μm. **F)** Analytical result of fibrotic area, * vs. other groups with different symbols (†, ‡, §, ¶), $p < .0001$. **G to K)** Illustrating the immunofluorescent microscopic finding (400) for identification of γ-H2AX (red color). Scale bars in lower right corner represent 20 μm. analytical result of number of γ-H2AX+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), $p < .0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 6$ for each group). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). CLI = critical limb ischemia; SC = sham control; ECSW = extracorporeal shock wave; SVF = stromal vascular fraction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

carotid ring angiogenesis and HUVEC angiogenesis in Matrigel assay between the two groups (Fig. 2).

3.2. SVF treatment suppressed the inflammatory reaction in in vitro study (Fig. 3)

The protein expressions of TNF-1α, NF-κB, MMP-9 and IL-1ß, four indicators of inflammation, were significantly increased in A7r5 + LPS than in other groups, significantly increased in A7r5 + LPS-SVF than in A7r5 only and A7r5 + SVF, but did not differ between the latter two groups.

3.3. Time courses of blood flow in CLI area (Fig. 4)

By day 1 after CLI procedure, laser Doppler scanning showed a significantly higher ischemic/normal blood flow (INBF) ratio in group 1 (SC) than in group 2 (CLI), group 3 (CLI + ECSW), group 4 (CLI + SVF) and group 5 (CLI + ECSW-SVF), yet no difference was shown among the latter four groups. By day 7 after CLI, the INBF ratio was significantly higher in group 1 than in other groups, significantly higher in groups 3 to 5 than in group 2, but it showed no difference among the groups 3 to 5. By day 14 after CLI, INBF ratio was highest in group 1, lowest in group 2, significantly higher in group 5 than in groups 3 and 4, but it showed no significant difference between groups 3 and 4.

3.4. Time courses of circulating level of endothelial progenitor cells (Fig. 5)

By day 0, flow cytometric analysis showed that the circulating levels of C-kit/CD31+, Sca-1/CD31+, CD34/KDR+ and VE-cadherin/CD34+ cells, four EPC surface markers, did not differ among the five groups. However, by days 5 and 14 after CLI procedure, the circulating levels of these parameters were progressively increased from group 2 to 1 and from groups 3 to 5.

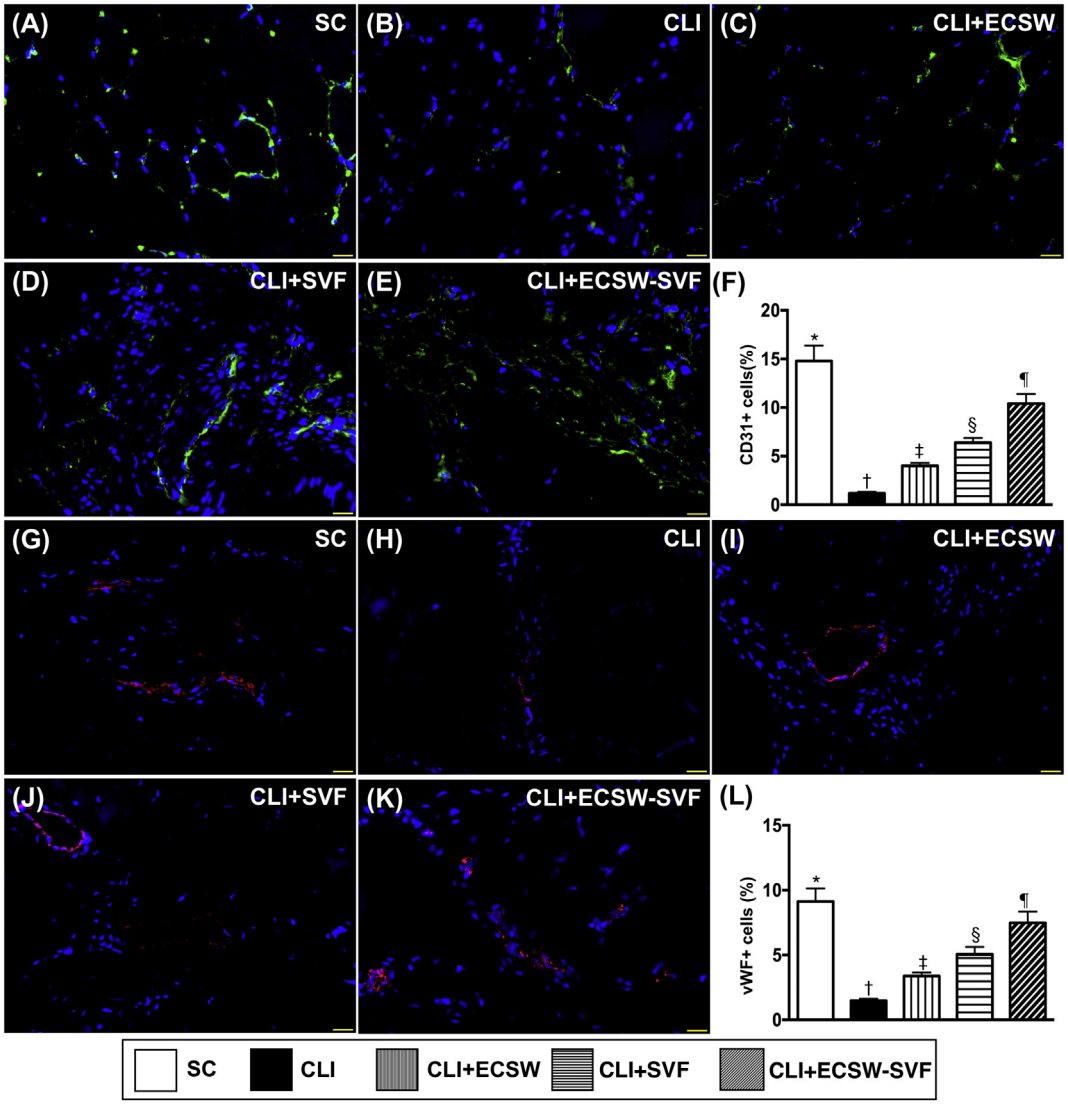

Fig. 9. Cellular expressions of endothelial cell markers in ischemic quadriceps muscle by day 14 after CLI procedure.
A to E) Illustrating immunofluorescent (IF) microscopic finding (400×) for identification of CD31+ cells (green color) in CLI quadriceps muscle. **F)** Analytical result of number of CD31+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), $p < .0001$. **G to K)** Illustrating the IF microscopic finding (400×) for identification of von Willebrand factor (vWF) + cells (red color) in CLI quadriceps muscle. **L)** Analytical result of number of vWF + cells, * vs. other groups with different symbols (†, ‡, §, ¶), $p < .0001$. Scale bars in lower right corner represent 20 μm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 6$ for each group). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). CLI = critical limb ischemia; SC = sham control; ECSW = extracorporeal shock wave; SVF = stromal vascular fraction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. The protein expressions of endothelial and angiogenesis biomarkers in ischemic quadriceps muscle by day 14 after CLI procedure (Fig. 6)

The protein expressions of CD31, vWF and eNOS, three indicators of integrity of endothelial cell/function, were significantly higher in group 1 than in other groups and significantly and progressively increased from groups 2 to 5. Additionally, the protein expressions of SDF-1α, VEGF and CXCR4, three indices of angiogenesis factors, were significantly and progressively increased from groups 1 to 5, implicating the increase in these parameters was an intrinsic response to ischemic stimulation and further increased in response to ECSW-SVF treatment.

3.6. Protein expressions of apoptotic, fibrotic and mitochondrial/DNA-damaged biomarkers in ischemic quadriceps muscle by day 14 after CLI procedure (Fig. 7)

The protein expressions of mitochondrial Bax, cleaved caspase 3 and cleaved PARP, three indicators of apoptosis, were significantly lower in group 1 than in other groups, and significantly and progressively reduced from groups 2 to 5. Consistently, the protein expression of Smad3 and TGF-ß, two indicators of fibrosis, and protein expression of γ-H2AX, an indicator of DNA-damaged biomarker, exhibited an identical pattern to apoptosis among the five groups. Additionally, the protein expression of cytosolic cytochrome C, an indicator of mitochondrial damage, displayed a similar pattern to that of γ-H2AX among the five groups. Conversely, the protein expression of mitochondrial cytochrome C, an indicator of mitochondrial integrity, displayed an opposite pattern of cytosolic cytochrome C among the five groups.

3.7. Histopathological findings of fibrotic area and DNA-damaged cell expression in ischemic quadriceps muscle by day 14 after CLI procedure (Fig. 8)

The Masson's trichrome stain demonstrated that the fibrotic area

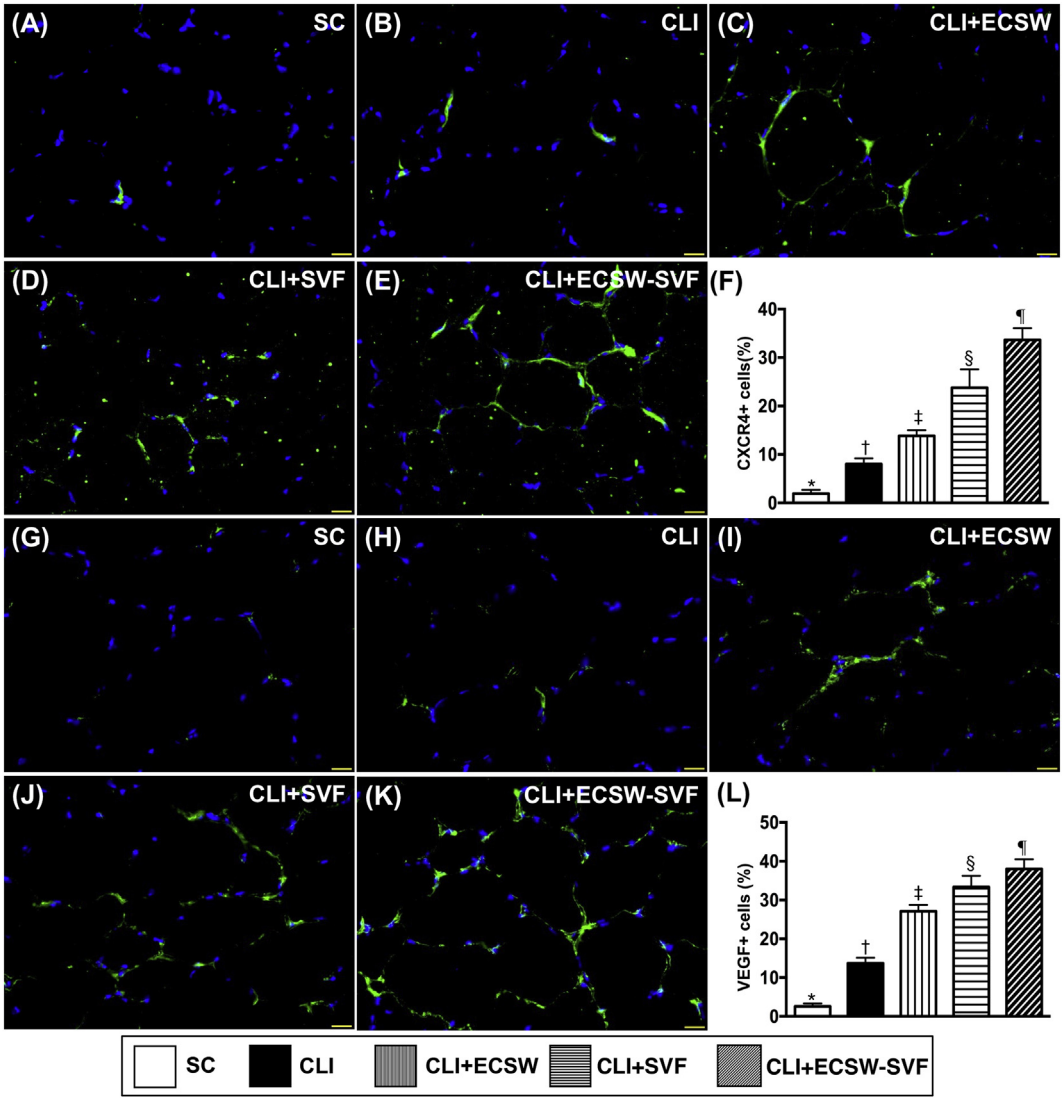

Fig. 10. Angiogenesis cell expressions in ischemic quadriceps muscle by day 14 after CLI procedure.
A to E) Illustrating immunofluorescent (IF) microscopic finding (400×) for identification of cellular expression CXCR4 (green color) in CLI quadriceps muscle. **F)** Analytical result of number of CXCR4+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), $p < .0001$. **G to K)** Illustrating the IF microscopic finding (400×) for identification of cellular expressions of vascular endothelial growth factor (VEGF) (green color) in CLI quadriceps muscle. **L)** Analytical result of number of VEGF + cells, * vs. other groups with different symbols (†, ‡, §, ¶), $p < .0001$. Scale bars in lower right corner represent 20 μm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 6$ for each group). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). CLI = critical limb ischemia; SC = sham control; ECSW = extracorporeal shock wave; SVF = stromal vascular fraction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was significantly lower in group 1 than in other groups, and significantly and progressively reduced from groups 2 to 5. Additionally, the cellular expression of γ-H2AX, an indicator of DNA damage, displayed an identical pattern to that of fibrosis.

3.8. The cellular expressions of endothelial cell markers in ischemic quadriceps muscle by day 14 after CLI procedure (Fig. 9)

The expressions of CD31+ and vWF+ cells, two indicators of endothelial cell integrity, were significantly higher in group 1 than in other groups, and significantly and progressively increased from groups 2 to 5.

3.9. Cellular expressions of angiogenesis cells in ischemic quadriceps muscle by day 14 after CLI procedure (Fig. 10)

The IF microscopic finding showed that the cellular expressions of CXCR4 and VEGF, two cellular angiogenesis markers, were significantly and progressively increased from groups 1 to 5.

3.10. The inflammatory biomarkers in ischemic quadriceps muscle by day 14 after CLI procedure (Fig. 11)

The cellular expression of F4/80, one cellular inflammatory biomarker, was significantly lower in group 1 than in other groups, and significantly and progressively reduced from groups 2 to 5. Additionally, the protein expressions of TNF-α and NF-κB, two indicators of inflammation, demonstrated an identical pattern of F4/80 inflammatory cells among the five groups.

3.11. Small vessel density in ischemic quadriceps muscle by day 14 after CLI procedure (Fig. 12)

The result of α-SMA stain demonstrated that the number of small

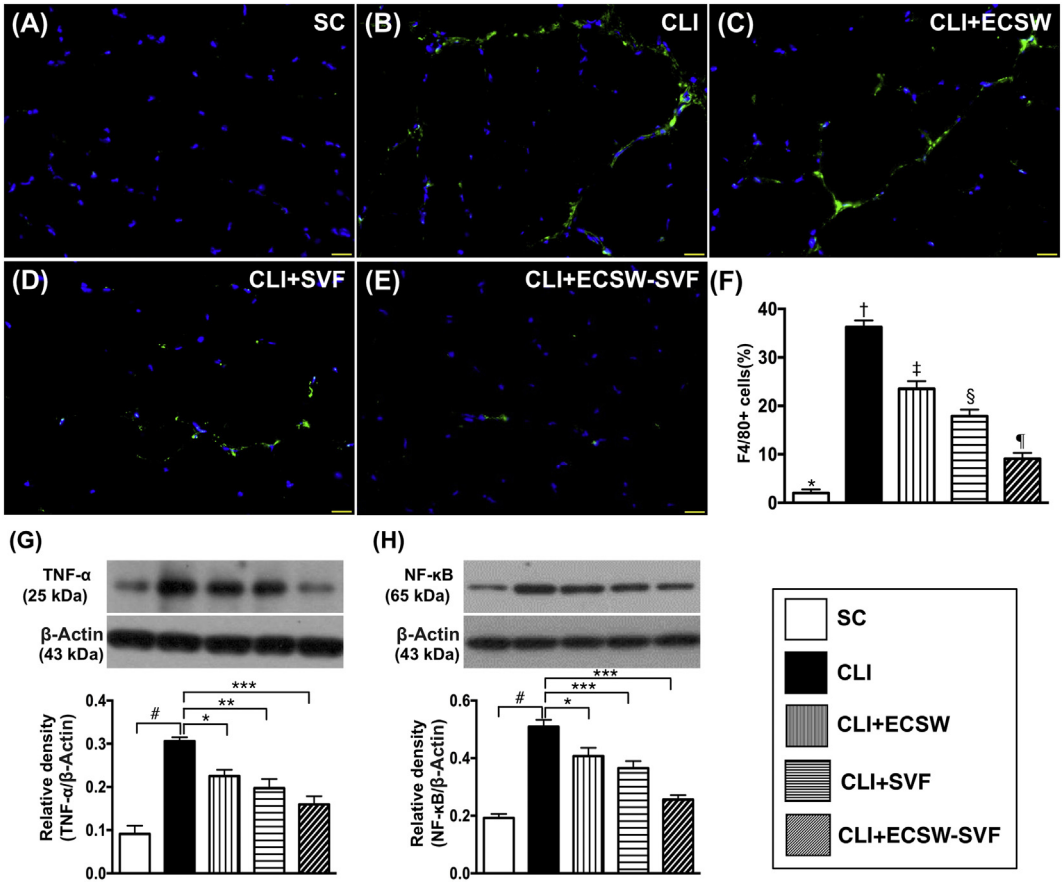

Fig. 11. Inflammatory biomarkers in ischemic quadriceps muscle by day 14 after CLI procedure.
A to E) Illustrating the immunofluorescent microscopic finding (400×) for identification of cellular expression of F4/80 (green color) in CLI quadriceps muscle. **F)** Analytical result of number of F4/80 + cells, * vs. other groups with different symbols (†, ‡, §, ¶). Scale bars in lower corner represent 20 μm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 6$ for each group). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). **G and H)** Protein expressions of tumor necrosis factor (TNF)-1α (G) and nuclear factor (NF)-κB (H). * indicated $p < .05$; ** indicated $p < .01$; *** indicated $p < .001$; # indicated $p < .0001$. CLI = critical limb ischemia; SC = sham control; ECSW = extracorporeal shock wave; SVF = stromal vascular fraction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

vessels (i.e., ≤25 μm) was significantly higher in group 1 than in other groups, and significantly and progressively increased from groups 2 to 5.

4. Discussion

This study which investigated the therapeutic impact of ECSW-SVF on CLI in rat yielded several striking implications. Frist, as compared with the CLI-only treatment, ECSW therapy significantly restored the blood flow in CLI. Our finding was consistent with the findings of our previous studies [29–31] that ECSW treatment significantly preserved ischemic related organ dysfunction and improved blood flow in ischemic organs. Second, therapeutic effect of SVF was comparable to ECSW, suggesting that autologous SVF is an alternative treatment for patients with CLI refractory to traditional therapy. Third, the combined ECSW-SVF therapy is superior to either one alone for enhancing angiogenesis and blood flow as well as protecting the quadriceps from CLI-induced injury, implicating this combination therapy may be a potential modality for CLI patients who are refractory to conventional treatment and also refuse to receive amputation for the CLI with gangrenous change.

By day 1 after CLI, the INBF ratio (i.e., blood flow in CLI region) was remarkably higher in SC (i.e., group 1) than in groups 2 to 5 (i.e., CLI with and without treatment), but this parameter did not differ among these four groups, implicating our experimental model of CLI was not only successfully created but also consistent for the purpose of individual study.

Our previous studies have shown that application of ECSW remarkably preserved ischemic related organ dysfunction and augmented blood flow in ischemic organs [29–31]. An important finding in the present study was that as compared with CLI animals, the blood flow in the CLI zone of animals was substantially restored after ECSW. Our finding, in addition to being consistent with the findings of previous studies [29–31], raises a consideration that this therapy may serve as an option for those patients with ischemic claudication/PAOD and refractory conventional treatment.

Currently, there is yet no report regarding the application of SVF for CLI patients. An essential finding in the present study was that as compared with ECSW, SVF treatment was found to be not inferior on restoring the blood flow in CLI. In this way, our finding, in addition to reporting the first therapeutic effect of SVF on restoring the blood flow in setting of CLI, established a non-inferior effect of SVF to ECSW on improving the ischemic event in the quadriceps muscle. Of distinctive finding in the present study was that combined ECSW-SVF therapy offered an additional benefit than either one alone on augmenting the blood flow in CLI area, highlighting that this combined therapeutic regimen may be potential for patients with severe CLI and the last resort of amputation is suggested by surgeon.

An association between ischemia and an increase in inflammation, oxidative stress, apoptosis and DNA/mitochondrial damage has been extensively investigated by a body of previous researches [29–31,36]. A principal finding in the present study was that the above-mentioned

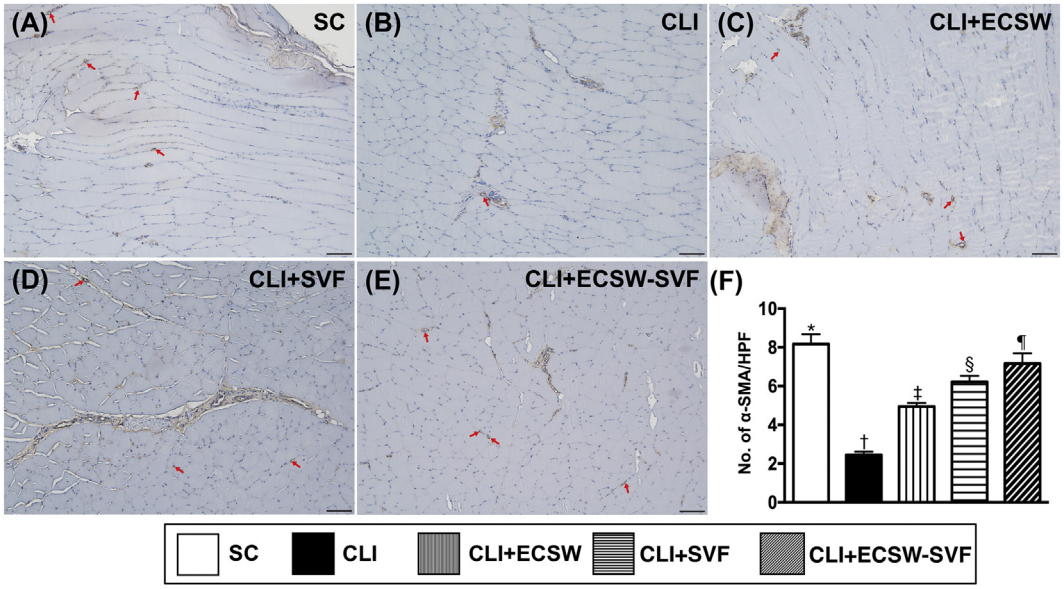

Fig. 12. Small vessel density in ischemic quadriceps muscle by day 14 after CLI procedure.
A to E) Illustrating the microscopic finding (100×) of α-smooth muscle actin (α-SMA) stain for identification of small vessel density (gray color) (red arrows) in CLI quadriceps muscle. **F)** Analytical result of number of small vessels (i.e., ≤25 μm), * vs. other groups with different symbols (†, ‡, §, ¶). Scale bars in lower right corner represent 100 μm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). CLI = critical limb ischemia; SC = sham control; ECSW = extracorporeal shock wave; SVF = stromal vascular fraction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

parameters were found to be remarkably increased in CLI animals as compared with SC counterparts. Our findings strengthened the findings from the previous studies [29–31,36]. Of important finding was that these aforementioned molecular-cellular perturbations were substantially downregulated by ECSW and SVF treatments. Furthermore, combined ECSW-SVF treatment was found to furthermore inhibit these molecular-cellular perturbations. These findings, at least in part, explain for why combined ECSW-SVF is better than either one alone for restoring the blood flow in CLI area and protecting the quadriceps muscle from CLI injury.

Angiogenesis/vasculogenesis has been clearly identified to play a crucial role for restoring the blood flow in ischemic region by previous studies [19,29–31,35]. A principal finding in the present study was that the protein and cellular angiogenesis biomarkers as well as number of small vessel density were significantly reduced in CLI animals than in those of the SC that were notably reversed in those of CLI animals by ECSW or SVF treatment and furthermore reversed by combined ECSW-SVF treatment. Our findings, in addition to reinforcing the findings of the previous studies [19,29–31,35], once again explained why the blood flow was significantly improved in CLI by ECSW-SVF treatment.

5. Study limitation

This study has limitations. Frist, the study period was relatively short, thus the long-term effect of ECSW-SVF therapy remains uncertain. Second, the sample size of each group in the present study was relatively small. Accordingly, the distortion of statistical significance could not be completely excluded. Third, our finding showed that ECSW-SVF therapy is superior to either one alone for restoring the blood flow in the ischemic area could be explained as a synergic effect of ECSW plus SVF therapy. On the other hand, although our result displayed that the blood flow in ischemic area did not differ between SVF and ECSW, we did not test the optimal dosages of ECSW or SVF in the present study. Thus, we could not conclude whether the therapeutic effect on restoring the blood in ischemic area was better in SVF than in ECSW or vice versa.

6. Conclusion

In conclusion, the results of the present study demonstrated that ECSW-SVF therapy is safe and effective on restoring the blood flow in setting of CLI in rodent. This finding highlight that the combined therapy may be the last resort without ethical problem for CLI patients at high-risk for PTA or bypass intervention.

Acknowledgements

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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