Pulsed Electromagnetic Fields Increase Angiogenesis and Improve Cardiac Function After Myocardial Ischemia in Mice

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Background: Previous studies have shown that pulsed electromagnetic fields (PEMF) stimulate angiogenesis and may be a potential treatment strategy to improve cardiac function after myocardial infarction (MI). This study explored the effects and its related mechanisms of PEMF in MI mice.

Methods and Results: MI mice were used in PEMF treatment (15 Hz 1.5 mT PEMF or 30 Hz 3.0 mT PEMF) for 45 min per day for 2 weeks. Furthermore, an in vivo Matrigel plug assay was used to observe the effect of PEMF in promoting angiogenesis. Compared with the sham PEMF group, PEMF treatment with 30 Hz 3.0 mT significantly improved heart function. PEMF treatment with 15 Hz 1.5 mT and 30 Hz 3.0 mT both increased capillary density, decreased infarction area size, increased the protein expression of vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 2 (VEGFR2), Ser473-phosphorylated Akt (pSer473-Akt) and S1177-phosphorylated endothelial nitric oxide synthase (pS1177-eNOS), and increased the mRNA level of VEGF and hypoxia inducible factor 1-alpha (HIF-1α) in the infarct border zone. Additionally, treatment with 30 Hz 3.0 mT also increased protein and mRNA level of fibroblast growth factor 2 (FGF2), and protein level of β1 integrin, and shows a stronger therapeutic effect.

Conclusions: PEMF treatment could promote angiogenesis of the infarct border zone and improve cardiac function in MI mice. A treatment parameter of 30 Hz 3.0 mT is remarkably effective in MI mice. The effect is associated with the proangiogenic signaling pathways of HIF-1α/VEGF/Akt/eNOS or HIF-1α/FGF2/Akt/eNOS.

Key Words: Angiogenesis; Cardiac rehabilitation; Electromagnetic fields; Ischemic heart disease; Myocardial infarction

Coronary artery disease (CAD) is the leading cause of morbidity and mortality globally.1 Massive myocyte death after ischemic episodes leads to compromised cardiac function, adverse left ventricular remodeling, and low quality of life in patients with CAD, highlighting the importance of revascularization or re-establishment of blood perfusion to the damaged myocardium.2 Although the development of the percutaneous coronary intervention (PCI) can improve coronary perfusion within regions of the ischemic myocardium, its clinical application is sometimes impeded by several limitations such as small vessel occlusion and the risks of restenosis and stent thrombosis.3 Therefore, stimulating collateral circulation through angiogenesis could be another strategy to enhance the blood supply to the hypoxic cardiomyocytes, and angiogenesis is now considered as a novel therapy for CAD.4

New therapeutic approaches to activate angiogenesis are being intensively studied. For instance, vascular endothelial growth factor (VEGF) protein and gene delivery have been demonstrated to be highly effective in promoting angiogenesis in preclinical models of chronic ischemia. However, it is clinically limited by its high cost and high technical requirement.5 Therefore, there is a need for a safe, effective, non-invasive and low-cost proangiogenic approach for ischemic disease.

Pulsed electromagnetic fields (PEMF), as a physical therapy, play an important role in medical fields. Over the last decades, large bodies of evidence have shown that PEMF may exert various therapeutic effects, including relieving pain and stiffness in osteoarthritis,6 protecting articular cartilage,7 controlling the development of osteoporosis,8 promoting regeneration of the bone and neuron,9,10 and facilitating skin wound healing.11

Previous studies have also revealed that PEMF can stimulate angiogenesis. Important functions of endothelial cells such as proliferation, migration, and tube formation are increased when the electric fields are applied in vitro.12 Moreover, in vivo studies showed that PEMF enhances microcirculation and angiogenesis in an animal ischemic disease model.13

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However, the optimal frequency and intensity that PEMF uses to promote angiogenesis and its mechanism are still unclear. Therefore, in this study, we used PEMF to intervene in mice ischemic myocardial to determine the frequency and intensity under which PEMF maximally promotes angiogenesis, and explore its relevant mechanisms.

**Methods**

**Animals and MI Model**

C57BL6 mice that were aged 10–12 weeks were used for all experiments. Provided by Chengdu Dashuo Inc., the mice were maintained in the Experimental Animal Center of the Science and Technology Base of West China Hospital of Sichuan University. All animals were kept in cages; 5 per cage, free to drink water and eat food. Light and dark time alternated for 12 h, and the animal room ambient temperature was 23±2°C. The animal study was approved by the Ethics Committee from the Animal Laboratory of Sichuan University. All institutional and national guidelines for the care and use of laboratory animals were followed.

A mouse MI model was established according to litera-
The trachea of the cannula is connected to a small animal ventilator. The left anterior descending branch of the coronary artery was ligated ~2 mm from the base of the left atrial appendage. If the color of the anterior wall of the left ventricle changes from bright red to pale, it is judged that the model is successful. The successful surviving mice of the model are randomly divided into 3 groups according to a randomized digital table.

**PEMF Exposure**

The magnetic field intervention device was developed by the Manufacturing College of Sichuan University. It consists of three parts: a pulse generator, a stepper motor driver, and a Helmholtz coil. The 1,000-turn copper wire (diameter 1 mm) forms a cylindrical solenoid around the plastic pipe. The coil has large magnetic field stability in the middle area, which can provide a uniform PEMF for the treatment of MI mice. In group 1, the frequency, intensity, and time of PEMF were 15 Hz, 1.5 millitesla (mT), and 45 min per day, respectively. In group 2, the frequency, intensity, and time of PEMFs were 30 Hz, 3.0 mT, and 45 min per day, respectively. In the control group, mice were treated with sham PEMF for 45 min by a dummy stimulator without delivering any output. PEMF treatment was started 1 day after surgery and continuous intervention for 2 weeks. Photographs of the established MI model and PEMF intervention device are shown in Supplementary Figure 1.

**Echocardiography**

Two-dimensional transthoracic directional M-mode and Doppler echocardiography was used to detect and calculate cardiac function of mice, including left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic shape.
diameter (LVESD), fractional shortening (FS), and ejection fraction (EF) before intervention and at 1 week and 2 weeks after intervention. Echocardiography was carried out by a specially assigned person who was blinded to the identity of the group. After anesthetizing the mice with 1% sodium pentobarbital solution, the ultrasonic gel was applied to the left chest where the hair was shaved, and echocardiography was performed.

**Tissue Collection**

After 2 weeks of continuous intervention, the mice were sacrificed, the hearts were removed by thoracotomy, and the blood of the hearts was washed away with phosphate buffered saline (PBS). Heart tissue for hematoxylin and eosin (HE) staining and immunohistochemistry were fixed in 4% paraformaldehyde solution. Heart tissues for triphenyl tetrazolium chloride (TTC) staining were quickly placed in a −80°C freezer. For Western blot and real-time polymerase chain reaction (real-time PCR) experiments, hearts were dissected to obtain the infarct border zone and then samples were snap-frozen in liquid nitrogen.

**TTC Staining**

TTC staining was performed to detect the extent of heart infarction. The heart tissues were frozen in a −80°C freezer for 20 min and serially sliced along the apex to the bottom of the heart. Then, the samples were immersed in a 0.5% TTC solution prepared in PBS, protected from light with aluminum foil, and placed in a 37°C constant temperature incubator for 15 min. The infarct area would be stained pale. The infarct area and total area of myocardium were measured, and the percentage of infarct area to total area was calculated.

**Immunohistochemistry**

Capillary density in the infarct border zone was determined by immunohistochemical staining of CD31. Fixed tissues were dehydrated, embedded in paraffin and sectioned, and then dewaxed and hydrated. Ethylenediaminetetraacetic acid (EDTA) was used for antigen retrieval. The sections were blocked with 3% hydrogen peroxide for 20 min at room temperature to block endogenous peroxidase. After incubating with rabbit serum at 37°C for 30 min, the CD31 primary antibody (R&D system) was added and incubated at 4°C overnight. The secondary antibody was added and incubated at 37°C for 1 h. The sections were then stained with 3,3′-Diaminobenzidine (DAB), the nuclei were stained with hematoxylin, and finally the samples were dehydrated, cleared, and sealed on slides. The sections were observed under a microscope (ZEISS Imager A2). Five 400× magnification fields of view were randomly selected from each slice to count the number of microvessels.

**Western Blot Analysis**

Myocardial tissue was lysed with radio immunoprecipitation assay (RIPA) buffer containing protease inhibitor and phosphatase inhibitor, and the protein content of the lysate was quantified by using a BCA kit. Samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in tris-buffered saline tween-20 (TBS-T) (20 mmol/L

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**Figure 4.** Pulsed electromagnetic fields (PEMF) improve Matrigel plug angiogenesis in vivo. (A-C) Representative photographs of immunofluorescence staining of CD31-positive cells in Matrigel plug slices. The upper right images of immunofluorescence show the Matrigel plug harvested at 14 days after the intervention. (D) CD31+ area was quantified by image J software. The results are presented as mean±SEM; **P<0.01, ***P<0.001.
Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h. The immunoblots were incubated with the primary antibody (anti-phospho Akt/eNOS or anti-Akt/eNOS/FGF2/VEGF/VEGFR2/β1 integrin) overnight at 4°C (the primary antibody against eNOS, Akt, phospho-Akt, β1 integrin and VEGFR2 were purchased from Cell Signaling Technology, phospho-eNOS from Abcam, VEGF from R&D Systems, and FGF2 from Absin), washed and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG) or a rabbit anti-goat IgG horseradish peroxidase. An enhanced chemiluminescent detection system was used for protein determination. Immunoreactive bands were quantified by densitometric analysis using the Image J software.

Real-Time PCR

The heart tissue frozen by liquid nitrogen was ground to a powder, which was then followed by the addition of trizol and chloroform. After centrifugation at 12,000 g at 4°C for 10 min, the supernatant liquid was extracted. Then, isopropanol was added to the supernatant liquid, and after standing for 10 min, it was centrifuged at 12,000 g again. The precipitate was washed with 75% alcohol and finally dissolved in sterile water treated with diethyl-pyrocarbonate (DEPC).

After cDNA was synthesized using the reverse transcription kit (Takara), real-time PCR was carried out using a CFX96 Real-time PCR detection System (Bio-Rad) and TB Green™ Premix Ex Taq™ II (Takara), following the manufacturer’s instructions. The primer sequences are shown in Table.

In Vivo Matrigel Plug Angiogenesis Assay and Immunofluorescence

Matrigel (BD Bioscience) was melted on ice overnight and then mixed with 300 ng/mL basic fibroblast growth factor (bFGF). After the mice were anesthetized with 1% sodium pentobarbital, matrigel (0.5 mL) was injected into the abdomens of mice aged 8–10 weeks. The mice were then

![Figure 5. Protein expression in the infarct border zone after pulsed electromagnetic fields (PEMF) intervention for 2 weeks. (A) Representative results of Western blot in 3 groups. (B) Quantitative analysis on FGF2, vascular endothelial growth factor (VEGF), VEGFR2, phosphorylated Akt, phosphorylated endothelial nitric oxide synthase (eNOS) and β1 integrin proteins. The levels from the control group were defined as 100%. The results are presented as mean±SEM; *P<0.05, **P<0.01, ***P<0.001.](image-url)
randomly divided into 3 groups based on the randomized digital table and were exposed to PEMF for 45 min per day for 2 weeks (Group 1: 15 Hz/1.5 mT treatment; group 2: 30 Hz/3.0 mT treatment; and group 3: 0 Hz/0 mT treatment). The mice were sacrificed at 2 weeks after the treatment, and the subcutaneous gels were isolated, embedded in optimal cutting temperature compound (OCT), and frozen in liquid nitrogen. The frozen plug was sectioned into 3-micron sheets using a cryostat. After being fixed with ice acetone, the sections were blocked with rabbit serum, and the sections were returned to the room temperature and incubated at 4°C overnight. The next day, anti-CD31 antibody (R&D system) was added before the sections were further incubated for another 5 min. The sections were observed under a microscope (ZEISS Imager Z2). Five 200× magnification fields of view were randomly selected for each section, and the areas of staining were analyzed and calculated with Image J.

Statistical Analysis
Data were presented as means±SEM. One-way analysis of variance (ANOVA) was used for statistical comparisons among groups. Dunnett’s post-hoc test is used for multiple comparisons between individual groups. A value of P<0.05 was considered statistically significant.

Results
PEMFs Improve Cardiac Function After MI
To determine whether PEMF promotes cardiac functions in MI mice, echocardiography was used prior to the intervention and 1 week and 2 weeks after the intervention. Echocardiographic studies showed the EFs (%) in the 30 Hz PEMF-treated group were significantly higher than those in the control group at 1 week and 2 weeks after intervention (P<0.01, P<0.05, respectively; Figure 1). Similarly, the FS (%) was increased in the 30 Hz PEMF-treated group (Figure 1).

PEMFs Decrease the Area of Infarction
After PEMF intervention for 2 weeks, a TTC assay was performed to evaluate the situation of infarcted myocardium (Figure 2A–C). And quantitative analyses showed that the infarct size of 15 Hz and 30 Hz in the PEMF-treated groups were smaller than the control group (P<0.05, P<0.001; Figure 2D).

PEMFs Improve Angiogenesis In Vivo
To confirm whether the changes in the myocardial function were associated with angiogenesis, immunohistochemistry staining of CD31 was carried out in the ischemic myocardium after the 2-week intervention. Quantitative analyses by counting the number of vessels revealed that capillary densities were significantly elevated in the 15 Hz and 30 Hz PEMF-treated groups as compared to those in the control group (P<0.001, P<0.001), and the effect in the 30 Hz PEMF-treated group is remarkably better than in the 15 Hz PEMF-treated group (P<0.001; Figure 3).

To substantiate our findings, a Matrigel plug angiogenesis assay was performed in vivo. Anti-CD31 immunofluorescence staining showed that the areas of blood vessels in the 15 Hz and 30 Hz PEMF-treated groups were significantly higher than those in the control group (P<0.001, P<0.001, respectively). And more blood vessels were observed in the 30 Hz PEMF-treated group (P<0.01) (Figure 4).

Potential Mechanism of PEMF Promoting Angiogenesis
The pro-angiogenic benefits of PEMF therapy drove us to investigate whether the angiogenesis was caused by activation of angiogenic signaling. To address the molecular mechanism by which PEMF promotes angiogenesis, we examined angiogenesis-related molecules at the protein (Figure 5) and mRNA levels (Figure 6). The 30 Hz, 3.0 mT PEMF intervention increased the protein expression of β1 integrin (P<0.05), fibroblast growth factor 2 (FGF2) (P<0.05), VEGF (P<0.01), and VEGFR2 (P<0.001) in MI mice, and the activations of Akt and eNOS, evidenced by more pSer473-Akt (P<0.05) and pS1177-eNOS (P<0.05) in the infarct border zone after the 2-week intervention compared with the control group. Similarly, the 15 Hz PEMF intervention increased the protein level of VEGF (P<0.01) and VEGFR2 (P<0.01) compared with the control group. The 30 Hz PEMF intervention in MI mice led to significantly higher protein levels of VEGFR2 (P<0.01) and p-Akt/Akt (P<0.05) as compared to that in the 15 Hz PEMF-treated group. The 30 Hz PEMF group significantly increased the mRNA levels of FGF2, VEGF, and HIF-α compared to

![Figure 6.](image-url)
those in the control group (P<0.05, P<0.05, P<0.05, respectively). However, in the 15 Hz PEMF-treated group, only the mRNA levels of VEGF and HIF-1α were increased (P<0.01, P<0.05). Western blot (Supplementary Figure 2) and histological analyses of the infarct area and remote area showed that PEMF will not cause pathological angiogenesis in non-ischemic areas.

Discussion

Our main findings are that: (1) PEMFs reduce infarct size and improve cardiac function in mice that have suffered myocardial infarction; and (2) this effect is associated with an increase in angiogenesis via the pro-angiogenic signaling of HIF-1α/VEGF/Akt/eNOS or HIF-1α/FGF2/Akt/eNOS.

After MI, the surviving left ventricular myocardium has an average hypertrophic response of 30%, while the coronary artery blood flow and vasodilation reserve of the surviving myocardium are both inhibited.56 Coordinated disruption of cardiac hypertrophy and angiogenesis after MI can lead to heart failure.17 Enhancing angiogenesis in ischemic myocardium is associated with a reduction of infarct expansion and restoration of cardiac performance, thereby preventing the development of heart failure.18,19 Reperfusion in the infarct border zone may salvage endocardial tissue and restore damaged myocardium. Reperfusion in this area with contraction-band necrosis may have greater tensile strength and less propensity to expand.20

Our results are in line with the observations from previous studies;21 that is, the ischemic myocardium with higher vascular density is associated with smaller infarct area and higher cardiac function, suggesting that the PEMF-induced cardioprotection may be partly due to increased blood perfusion to the ischemic myocardium.

We observed that PEMF treatment enhanced angiogenesis both in the infarct border zone and in the Matrigel plug, which may explain the cardioprotective effects of PEMF. There is mounting evidence that PEMFs facilitate proliferation, migration, and tube formation of endothelial cells, thereby enhancing angiogenesis.22,23 One possible mechanism responsible is that PEMF causes voltage-gated calcium influx into the cells, which might act as the signal for the cellular responses associated with angiogenesis.24 In the present study, we demonstrated that PEMFs upregulate HIF-1α and its downstream growth factors, VEGF and FGF2, which are two well-established, potent angiogenic activators.25 Moreover, the increased levels of phosphorylation of Akt and eNOS in endothelial cells are associated with improved survival and migration, thereby augmenting angiogenesis.26,27

It has been reported that integrin may serve as a mechanical sensor of low frequency electric field,28 which can convert physical force into a biochemical signal event.29 Some studies have reported that electric field exposure promotes cell migration30 and upregulates the protein expression of β1 integrin.31 The largest group of integrins. The low energy shock wave (SW) therapy significantly increased the protein level of β1 integrin.32 Knockdown of β1 integrin suppressed the SW-induced or low-intensity pulsed ultrasound (LIPUS)-induced phosphorylation of Akt and upregulation of VEGF.31,33 Therefore, the beneficial effects of electric field therapy, SW therapy and LIPUS therapy are related to β1 integrin.31,33 The role of β1 integrin in angiogenesis has become an important investigation subject, which regulates sprouting, branching, adhesion and cell survival.34,35 In our study, the protein expression of β1 integrin increased after 30 Hz, 3.0 mT PEMF intervention. Therefore, the effect of physical force on angiogenesis may be related to integrin.

In recent years, there have been many studies of non-invasive angiogenic therapy on ischemic heart disease. Low energy SW therapy has been demonstrated to upregulate VEGF expression in vivo and vitro,36 enhance angiogenesis and improve heart function of animal models36–38 and patients with MI.39 Low-intensity pulsed ultrasound (LIPUS) therapy has also been demonstrated to increase capillary density in a pig model of chronic myocardial ischemia40 and improve left ventricular remodeling after MI in mice.33 So far, 2 articles have reported the effects of PEMF on myocardial infarction in a rat model. In 2010, Yuan et al reported that 15 Hz/6 mT could promote myocardial angiogenesis and improve cardiac function after MI in rats through the VEGF/VEGF-R2 signaling pathway, whereas 10 Hz/4 mT cannot.41 In 2014, Hao et al reported that 30 Hz/5 mT could induce angiogenesis and preserve cardiac systolic function after MI in rats through activating the VEGF-eNOS system.42 The PEMF treatment may have the so-called “window effect”; that is, the biological responses occur within specific ranges of field frequency and amplitude of the PEMF.43 It was reported that PEMF treatment at 30 Hz, 5 mT, 32 min per day for 3 weeks had an anti-apoptosis effect on the cardiomyocytes.44

In the present study, we observed increases of angiogenesis in both PEMF-treated groups, with a more significant increase in the 30 Hz/3.0 mT group than in the 15 Hz/1.5 mT group. These data suggest that different treatment parameters of PEMF, including frequency, intensity, and duration, may exert different effects through different mechanisms. In this regard, it is necessary for future studies to further investigate the optimal parameters of the PEMF treatment.

The PEMF-induced cardioprotective effects may not simply be explained by the increased angiogenesis. Other potential mechanisms that also contribute to the therapeutic effects of PEMFs, such as enhancing cardiomyocyte survival, need to be investigated.

In conclusion, our study demonstrated that 15 Hz/1.5 mT and 30 Hz/3.0 mT PEMFs limit infarct expansion and preserve heart function in mice following MI. It seems that the latter condition had a more significant or beneficial effect compared to the former one. The PEMF-induced benefits probably result from the augmentation of angiogenesis in or near the infarcted myocardium via the activation of the HIF-1α/VEGF and HIF-1α/FGF2 signaling pathway. Therefore, PEMF is a promising approach to the treatment of ischemic heart disease.

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Disclosure

The authors report no conflicts of interest.


**Supplementary Files**