Original article

Enhanced external counterpulsation promotes growth cytokines-mediated myocardial angiogenesis in a porcine model of hypercholesterolemia

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Background Enhanced external counterpulsation (EECP) improves ischemia in patients with refractory angina pectoris, but the mechanism remains unclear. To explore the mechanisms of EECP action, we detected progenitor cells presenting any of the following markers $CD34^+$, $CD29^+$, and $CD106^+$.

Methods Growth cytokines-mediated progenitor cell mobilization and associated angiogenesis potential were assessed in a porcine model of hypercholesterolemia. Twenty-four male domestic swines were randomly assigned to 4 groups: normal diet (control, *n*=6), hypercholesterolemic diet (CHOL, *n*=6), hypercholesterolemic diet with administration of recombinant human granulocyte colony-stimulating factor (rhG-CSF) (rhG-CSF, *n*=6), and hypercholesterolemic diet with EECP treatment (EECP, *n*=6). EECP was applied 2 hours every other day for a total of 36 hours. Serum levels of vascular endothelial growth factor (VEGF) and granulocyte colony-stimulating factor (G-CSF), peripheral blood progenitor cell counts, level of regional angiogenesis, and expression of VEGF and stromal cell derived factor 1α (SDF-1α) in porcine myocardium were assessed, respectively.

Results A porcine model of hypercholesterolemia-induced arteriosclerosis was successfully established. There was no significant difference in serum levels of VEGF among the four groups. The serum levels of G-CSF in the EECP group increased significantly at week 15 and week 18 ((38.3±5.6) pg/ml at week 15 vs (26.2±3.7) pg/ml at week 12, P <0.05, and (46.9±6.1) pg/ml at week 18 vs (26.2±3.7) pg/ml at week 12, P <0.01). The serum levels of G-CSF in group 3 increased also significantly after receiving rhG-CSF injection for five days ((150±13.9) pg/ml at week 18 vs (24.8±5.4) pg/ml at week 12, P <0.01). Compared to other groups and other time points, progenitor cell counts increased significantly after 2-hour EECP treatment (108±13 vs 26±6 per 10^5 leukocytes, P <0.01), but not at week 18. The progenitor cell counts also increased significantly after subcutaneous injection of rhG-CSF for five days compared to the week 12 (baseline) (180±21 vs 25±7 per 10^5 leukocytes, P <0.01). There was no significant difference among the four groups at other time points. Moreover, the expression of VEGF and SDF-1α and the level of regional angiogenesis in myocardium increased significantly in both EECP and rhG-CSF groups.

Conclusions The results demonstrated that EECP could facilitate angiogenesis in the myocardium of atherosclerotic swines by increasing endogenous G-CSF, inducing an enhanced mobilization of progenitor cells and augmenting myocardial expression of VEGF and SDF-1α.

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Since the first report published in 1973, enhanced external counterpulsation (EECP) has been proved to be effective for the treatment of coronary artery disease (CAD) by enhancing diastolic coronary blood flow that increases endothelial shear stress, which in turn activates the release of various angiogenic cytokines to promote angiogenesis, consequently improving endothelial function.[1-3] Our previous study in a chronic canine model of coronary occlusion proved that EECP provided effective augmentation of diastolic coronary blood flow and an increased myocardial perfusion pressure, as well as a formation of new cardiac microvessels and relevant vascular endothelial growth factor (VEGF) expression.[4] Barsheshet et al[5] also demonstrated that EECP could increase the colony-forming capacity of circulating endothelial progenitor cells in patients with angina; however, it was still uncertain which kind of progenitor cells got involved in the therapeutical benefits of EECP. To explore the mechanisms of EECP action, we detected

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progenitor cells presenting any of the following markers $CD34^+$, $CD29^+$, and $CD106^+$, as identified by flow cytometry. $CD34^+$ cells are considered to be of hematopoietic lineage. $CD29^+$ and $CD106^+$ cells could represent bone marrow mesenchymal stem cells which possess a broader intrinsic pluripotent differentiation potential, including angiogenesis.[6-8]

METHODS

Development of porcine hypercholesterolemic model

This study was approved by the Animal Research Facility at Sun Yat-Sen University and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised in 1996). Twenty-four male domestic swines, with 20 days of age and weighed (7.55± 0.41) kg, were randomly assigned to 4 groups: group 1 (*n*=6, control), fed with a normal chow diet; group 2 (*n*=6, CHOL), fed with hypercholesterolemic chow diet, as previously described;[9] group 3 (*n*=6, rhG-CSF), fed with hypercholesterolemic diet plus subcutaneous injection of recombinant human granulocyte colony-stimulating factor (rhG-CSF) in the last five days; group 4 (*n*=6, EECP), fed with hypercholesterolemic diet plus EECP treatment. While receiving EECP treatment, group 4 was given continued hypercholesterolemic diet similar to the groups 2 and 3.

Blood sample collection and peripheral progenitor cell detection

Peripheral blood samples were collected from the animals at baseline, week 12 and week 18 for the determination of VEGF and granulocyte colony-stimulating factor (G-CSF). The blood samples were collected in the morning prior to EECP and 2 hours immediately post EECP treatment at week 12 and week 18 for peripheral progenitor cell count by flow cytometry. The antibodies were R-phycoerythrin (PE)-conjugated mouse anti-human CD34 monoclonal antibody, purified mouse anti-pig CD29 monoclonal antibody (BD Biosciences Pharmingen, USA) and anti-porcine CD106 (Antigenix America Inc., USA). To phenotypically characterize peripheral progenitor cells, fluorescein isothiocyanate (FITC)-or PE-conjugated monoclonal antibodies specific for CD34, CD29 and CD106 (BD Biosciences Pharmingen) were used.

Assessment of porcine model of hypercholesterolemia

For the assessment of the successful porcine model of hypercholesterolemia, the aortic portion at abdomen was longitudinally incised and fixed in 10% neutral formaldehyde for 48 hours, then stained in Sudan III to detect endovascular lipid deposition. Fatty streak and atherosclerotic plaque were photographed and analyzed with the Zeiss-KONTRON IBAS 2.0 Image Processing System for the quantitative studies of grey scanning and percent area of Sudan-III-positive area over the total area of the vascular endothelium.

EECP protocol and rhG-CSF administration

As described before,[9] EECP was given two hours every other day up to a total of 36 hours in group 4. The rhG-CSF (Hangzhou Jiuyuan Gene Engineering Co., Ltd., China) was subcutaneously administrated at the dose of 5 $\mu g \cdot kg^{-1} \cdot d^{-1}$ in the last five days of the study in group 3.

Assessment of arterial shear stress in pigs receiving EECP treatment

For the calculation of arterial wall shear stress in pigs receiving EECP treatment, internal diameter (ID) and blood flow velocity (V) at the right brachial artery (RBA) were measured just prior to and during EECP treatment, according to a previous study.[9]

Myocardial tissue sample collection and preparation

At the end of week 18, all animals were sacrificed by intravenous injection of a lethal dose of 10% potassium chloride and the hearts were excised. Myocardial tissues were harvested from the region of the left ventricle between the left anterior descending artery (LAD) and the circumflex artery (LCX). Each tissue sample was assigned to two parts. The first part was fixed in 10% buffered formalin and then sectioned for the study of morphology and immunostaining. The second part was immediately frozen in liquid nitrogen and then stored in –80°C for detecting VEGF and stromal cell-derived factor 1α (SDF-1α) mRNA expression by reverse transcription polymerase chain reaction (RT-PCR) and protein expression by Western blotting.

Morphological study and immunohistochemical analysis

Formalin-fixed mid-layer myocardial tissues taken from the left ventricle between the LAD and LCX were embedded in paraffin and cut into 4 μm transmural sections for immunostaining of microvessels. The antibodies were anti-human actin monoclonal antibodies (Maixin-Bio CO., Ltd., Fuzhou, China). Positively stained areas were quantitatively measured from 5 randomized microscopic fields (100×) using computer-assisted morphometry by Image-Pro plus 5.1 (Media Cybernetics, Inc., USA). Positively stained microvessels were defined as round structures with a central lumen containing a monolayer of smooth muscle cells that stained positively for α-actin. Microvessels showing positive staining for α-actin were counted under a microscopy with magnification by 100 times. The number of microvessels identified from 5 observed fields in each section was averaged and expressed as "numbers per square centimeters". Investigators performing histological and immunohistochemical analysis were blinded to experimental animals.

RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer′s instructions. The sequences of specific primers were as follows: VEGF forward, 5′-TCTCTTGGGTGCATTGG-

AGC-3′, reverse, 5′-CCTTGGTGAGGTTTGA-TCCG-3′. SDF-1α forward, 5′-CCTTGCCGATTCTTTGAGAGC-3′, reverse, 5′-GGAAATAAACATCCCGCCGT-3′. As an endogenous internal standard control, the levels of β-actin expression were also analyzed using the following primers: forward, 5′-GAAGATCCTCACGGAGCGG-3′, reverse, 5′-CCACACGGAGTACTTGCGC-3′. For the semi-quantification, each PCR product band was captured, and the intensity of expression was quantitated using an AlphaImager gel analysis system (Alpha Innotech, San Leandro, CA, USA). β-actin mRNA expression served as an internal standard.

Western blotting

The myocardial tissues frozen at –80°C were lysed in modified radioimmunoprecipitation buffer containing protease and phosphatase inhibitors. The samples for detecting VEGF and β-actin were subjected to 10% SDS-polyacrylamide electrophoresis. The samples for SDF-1α were subjected to 15% SDS-polyacrylamide electrophoresis. Blots were incubated with the primary mouse anti-VEGF monoclonal antibody and β-actin and rabbit anti-human SDF-1α affinity purified polyclonal antibody (Chemicon) at 4°C for 12 hours. Reactive protein was detected using the ECL chemiluminescence system (Pierce, Rockford, USA).

Statistical analysis

Data are presented as means ± standard deviation (SD). Statistical significance was evaluated by analysis of variance (ANOVA), followed by Scheffé′s procedure and by repeated ANOVA to test for interactions. P <0.05 was considered statistically significant.

RESULTS

A porcine model of atherosclerosis was successfully developed by hypercholesterolemic diet. As shown in Figure 1, atherosclerotic lesion was evaluated by Sudan III staining of the abdominal aorta. Atherosclerotic lesion was successfully developed by hypercholesterolemic diet for 12 weeks (Figure 1A). The positive Sudan III staining area (%), indicating atherosclerosis formation, was significantly increased in animals of CHOL group while comparing with that of the control ((9.18±3.76)% vs (0.89±0.35)%, P <0.05) (Figure 1B).

During the treatment of EECP, the peak diastolic arterial shear stress was significantly enhanced comparing with the pre-EECP condition ((45.91±14.10) dyne/cm^2 vs (22.13±7.17) dyne/cm^2), P <0.05), while the peak systolic arterial shear stress was also slightly increased during EECP ((43.71±12.29) dyne/cm^2 vs (39.71±16.72) dyne/cm^2, P >0.05).

There was no significant difference in serum levels of VEGF among the four groups at different time points. The serum levels of G-CSF in the EECP group increased significantly at week 15 and week 18 ((38.3±5.6) pg/ml at week 15 vs (26.2±3.7) pg/ml at week 12, P <0.05, and (46.9±6.1) pg/ml at week 18 vs (26.2±3.7) pg/ml at week 12, P <0.01). The serum levels of G-CSF in group 3 increased also significantly after receiving rhG-CSF injection for five days ((150±13.9) pg/ml at week 18 vs (24.8±5.4) pg/ml at week 12, P <0.01).

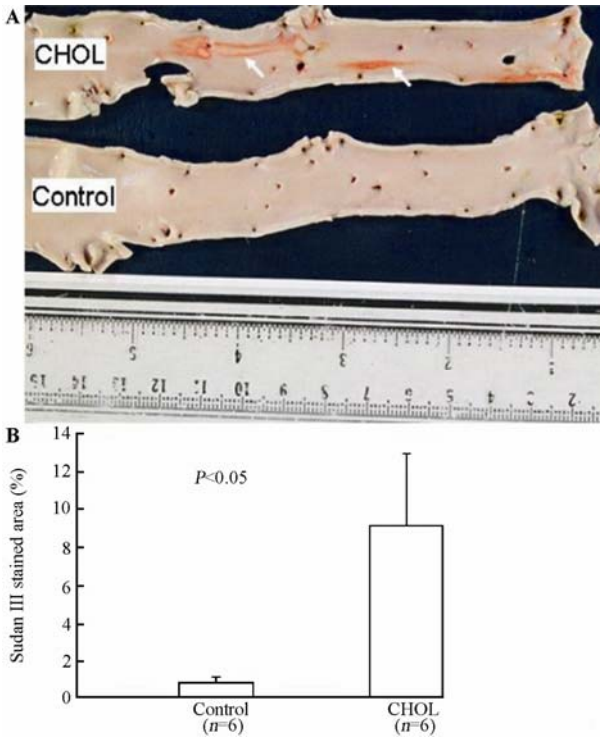


Figure 1. Atherosclerotic lesion of the porcine abdominal aorta developed by hypercholesterolemic diet. The solid white arrow indicates atherosclerotic lesion which was stained by Sudan III dye in color pink (**A**). The Sudan III stained area (%) was expressed by counting the positive area in total intimal area and averaged from all animals. Quantitative analysis demonstrated the atherosclerotic lesion was significantly promoted by hypercholesterolemic diet indicating a successful atherosclerotic model (**B**, P <0.05).

The progenitor cell counts in peripheral blood were shown in Figure 2. Compared to other groups and other time points, progenitor cell counts increased significantly after 2-hour EECP treatment (108±13 vs 26±6 per 10^5 leukocytes, P <0.01), but not at week 18. The progenitor cell counts also increased significantly after subcutaneous injection of rhG-CSF for five days compared to the week 12 (baseline) (180±21 vs 25±7 per 10^5 leukocytes, P <0.01). There was no significant difference among the four groups at other time points.

The expression of mRNA of VEGF and SDF-1α in cardiac tissue was demonstrated in Figure 3. The expression of both growth cytokines in the left ventricular myocardium was increased significantly after EECP treatment, compared to other groups (Figure 3A and 3B). Meanwhile, the protein expression of VEGF and SDF-1α in the cardiac tissue was also increased significantly after EECP (Figure 3C and 3D).

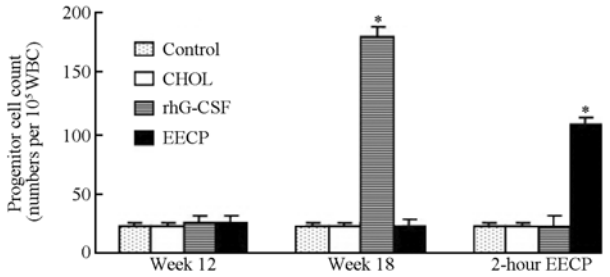


Figure 2. Peripheral progenitor cell counts. Compared to week 12, peripheral progenitor cell counts increased significantly in animals with administration of rhG-CSF injection at week 18. For animals treated with EECP, there was also a significant increase of progenitor cell counts after 2-hour of EECP therapy, but no time-dependent response was observed at week-18 ($^*P$ <0.01 vs week 12).

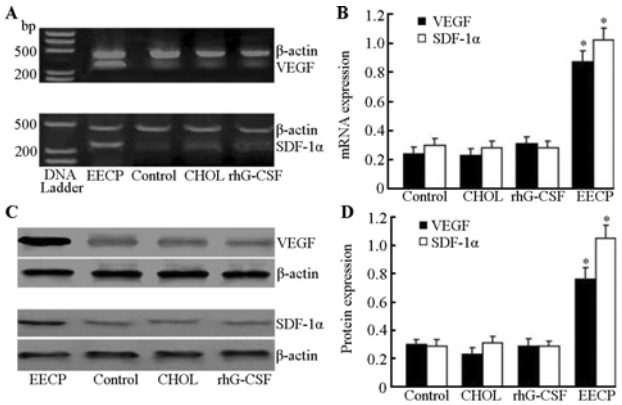


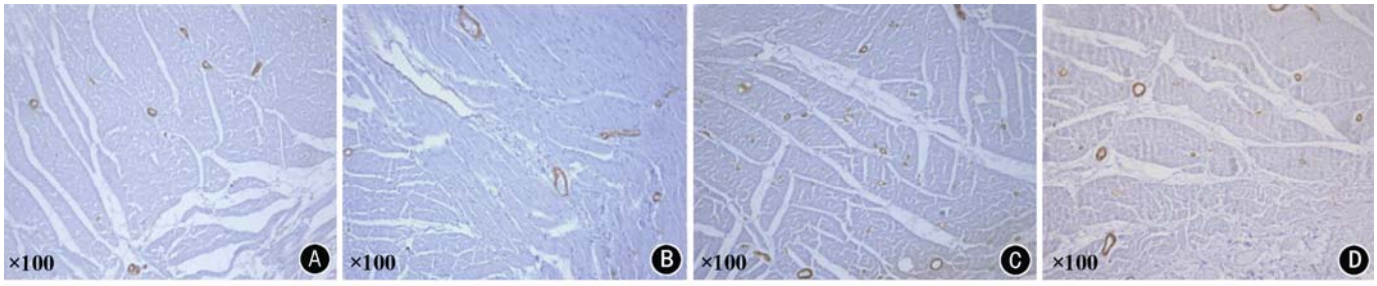
Figure 3. Expression of VEGF and SDF-1α at both mRNA (**A**) and protein (**C**) level, and its quantitative analysis of mRNA (**B**) and protein (**D**) expression. $^*P$ <0.01 vs other three groups. α-actin served as an internal standard for each sample, densitometry of VEGF and SDF-1α mRNA and protein expression were normalized by β-actin, and averaged value was obtained through all cases of each group.

The microvessels developed in cardiac tissues were shown in Figure 4 A–D. The positive stained spots by anti-α-actin staining were significantly higher in the porcine myocardium treated with EECP than that without EECP (CHOL group) (P <0.01) (Figure 4E), indicating that more microvessels were formed in the EECP group in response to the treatment. A corresponding increase of microvessel formation was also observed in pigs receiving rhG-CSF injection (P <0.05).

DISCUSSION

Enhanced external counterpulsation (EECP) is a potential treatment that is safe, noninvasive and technically simple. It has been demonstrated that EECP is beneficial in the treatment of patients with coronary artery disease by improving angina, quality of life (QOL), exercise tolerance, time to ST-segment depression during exercise stress, myocardial perfusion at rest and stress, and dobutamine stress-induced regional wall motion abnormality in patients with refractory angina. The sustained symptomatic and QOL benefit can be achieved in most patients for up to 3 years.[10] However, the underlying mechanisms of the above benefits still remain unknown. Our previous animal studies demonstrated that EECP could not only generate enhanced diastolic augmentation and myocardial perfusion, but lead to more microvessel formation and VEGF release at peri-infarct myocardium in dogs with myocardial infarction.[4] The present study for the first time investigated the efficacy of EECP therapy on myocardial angiogenesis in a swine model of hypercholesterolemia and atherosclerosis.

As demonstrated by many studies, low endothelial shear stress (ESS) is closely associated with atherosclerosis by attenuating nitric oxide (NO)-dependent atheroprotection, promoting low-density lipoprotein cholesterol uptake, synthesis, permeability and oxidative stress. Variations in local intravascular hemodynamic environment over time lead to dynamic interactions with arterial wall.[11] Up to date, there is no other way to enhance arterial wall shear stress in patients with angina pectoris except physical exercise; however, recent data from EECP studies support the hypothesis that increasing shear stress possibly represents one of the major mechanisms by which EECP exerts its clinical benefit.[9] Increases in shear stress may



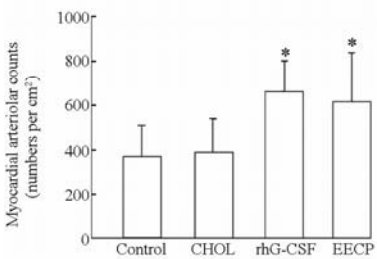


Figure 4. Representative immunostaining of α-actin within four groups. The bar graph presented quantitative analysis of α-actin. $^*P$ <0.01 comparing to the CHOL group. **A:** Control. **B:** CHOL. **C:** rhG-CSF. **D:** EECP.

improve endothelial function, suppress apoptosis in endothelial cells and facilitate angiogenesis.[9,12,13] It is inducible that increased shear stress activates complex intracellular signaling cascades and in turn, up-regulates many cytokines and subsequently promotes angiogenesis or arteriogenesis.[1,13,14] In our study, the peak diastolic arterial shear stress was significantly augmented by 2 folds during EECP treatment. Physiologically, the flow velocity of the coronary artery in diastolic phase is very low at rest. The enhanced shear stress at the level of 45 dyne/cm^2 during EECP is quite comparable to the peak systolic arterial shear stress and also the peak diastolic arterial shear stress during the exercise.[11,15,16] EECP treatment facilitates myocardial angiogenesis, suggesting that one of the beneficial effects of EECP on coronary arterial atherosclerotic disease might relate to increasing arterial shear stress and local microvessels, which further improves effective blood perfusion within ischemic myocardium, and consequently improves myocardial performance.

It is known that stem cells can be mobilized into peripheral blood by a variety of factors including tissue trauma, ischemia, proangiogenic factors and increased flow shear stress, and then migrate to injured tissues.[17,18] The proangiogenic factors include VEGF, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, SDF-1α, and other underlying cytokines. Previous studies have demonstrated the stimulation or up-regulation of coronary angiogenesis via triggering of the VEGF signaling pathway.[19-24] Blockage of VEGF or its receptor has been shown to cause inhibition of angiogenesis.[25]

GM-CSF and G-CSF have similar efficacy to induce monocyte mobilization, migration and proliferation, thereby facilitating cardiac regeneration and microvessel formation.[26-28] GM-CSF pretreatment to mobilize endothelial progenitor cells could induce capillary formation and enhance blood pressure ratio in ischemic or normal hindlimbs that suggests a corresponding increase in hindlimb blood flow.[17] The local chemokine SDF-1α can mobilize and guide stem cells migrating towards their downstream targets and further promote angiogenesis.[29-31]

In the present study we demonstrated that, compared to baseline, the levels of serum G-CSF increased significantly after 18 and 36 hours of EECP treatment, and the expression of mRNA and protein of VEGF and SDF-1α in cardiac tissue increased significantly after 36 hours of EECP treatment. These findings suggest that the benefit of EECP in facilitating myocardial angiogenesis on hypercholesterolemic and atherosclerotic swine might be associated with the up-regulation of relevant cytokines in peripheral circulating blood and regional cardiac tissue that could in turn stimulate angiogenesis of the myocardium.

Shear stress could increase the expression of the vascular endothelial cell-specific markers of stem cells at both the protein and the mRNA levels, indicating that shear stress selectively promotes the differentiation of stem cells into the endothelial cell lineage.[13,32] Flow shear stress has also been demonstrated to mediate stem cell mobilization and expression of angiogenic cytokines.[32,33] One of underlying mechanisms of EECP is to promote arterial wall pulsatile shear stress besides improving myocardial ischemia.[1,3,9] In the present study, a dose-response relation between EECP therapy and serum VEGF levels was not demonstrated. However, enhanced VEGF expression at both mRNA and protein levels in local myocardium was demonstrated after EECP treatment in contrast to the control group, and this phenomenon was also documented by our previous study in dogs.[4] One possible explanation is that EECP predominantly affects the coronary circulation by augmenting diastolic pulsatile flow and pressure gradient, promoting conditions for VEGF production in the microcirculatory environment of the myocardium. Increased circulating VEGF concentrations may have been transient, below detectable levels, or localized and thus not measurable in serum. Further studies are needed to explore these speculations.

Interestingly, we found that peripheral progenitor cell counts significantly increased immediately post 2-hour EECP treatment, but not at week 18. That may indicate an acute and transient effect of EECP action for peripheral progenitor cell mobilization during the treatment, and this effect on progenitor cell mobilization could not last for 48 hours. However, frequent stimulation for progenitor cell mobilization by EECP for 2 hours every other day might be strong enough to promote myocardial angiogenesis and microvessel formation. The resultant angiogenesis of EECP therapy may further imply the complex mechanisms rather than G-CSF stimulation *per se*. It has been confirmed that, apart from other effects, EECP may mobilize progenitor cells into peripheral blood, which is consistent with previous findings and further contributes, at least in part, to angiogenesis.[13,32]

In summary, the present study shows that EECP could facilitate angiogenesis in hypercholesterolemic and atherosclerotic porcine myocardium by increasing endogenous G-CSF and enhancing stem cell mobilization via up-regulation of myocardial VEGF and SDF-α at both mRNA and protein levels. These findings explain, at least in part, the beneficial effects of EECP treatment on atherosclerotic cardiovascular diseases. However, the long-term outcome and the underlying signaling pathway remain poorly understood. Further studies are underway to assess detailed cellular/molecular mechanisms of EECP on facilitating cardiac angiogenesis in the treatment of CAD.

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