Angiogenic effects of long-term enhanced external counterpulsation in a dog model of myocardial infarction

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Enhanced external counterpulsation (EECP) is an effective noninvasive treatment of coronary artery disease. Its mechanism of action remains unknown. An acute coronary occlusion dog model was created to explore the angiogenic effect of EECP. After coronary occlusion, 12 dogs were randomly assigned to either EECP (n = 6) or control (n = 6). Immunohistochemical studies of α-actin and von Willebrand factor (vWF) were used to detect newly developed microvessels. Systemic and local vascular endothelial growth factor (VEGF) were identified by ELISA and reverse transcriptase PCR analysis. There was a significant increase in the density of microvessels per squared millimeter in the infarcted regions of the EECP group compared with the control group (vWF, 15.2 ± 6.3 vs. 4.9 ± 2.1, P < 0.05; α-actin, 11.8 ± 5.3 vs. 3.4 ± 1.2, P < 0.05). The positive-stained area per squared micrometer also increased significantly (α-actin, 6.6 × 10^3 ± 2.9 × 10^3 μm^2 vs. 0.6 × 10^3 ± 0.5 × 10^3 μm^2, P < 0.05; vWF, 5.7 × 10^4 ± 1.9 × 10^4 μm^2 vs. 1.7 × 10^4 ± 1.4 × 10^4 μm^2, P < 0.05). Immunohistochemical staining and reverse transcriptase PCR analysis documented a significant increase in VEGF expression. These factors associated with angiogenesis corresponded to improved myocardial perfusion by 99mTc-sestamibi single-photon emission computed tomography. Angiogenesis may be a mechanism of action for the improved myocardial perfusion demonstrated after EECP therapy.

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Am J Physiol Heart Circ Physiol 290: H248–H254, 2006. First published August 19, 2005; doi:10.1152/ajpheart.01225.2004.—Enhanced external counterpulsation (EECP) has been demonstrated to be effective in the treatment of patients with coronary disease. A randomized clinical trial demonstrated significant improvement in the time to ST-segment depression during treadmill stress tests in the EECP-treated group compared with the control group (1). Several case study series and the International EECP Patient Registry, with more than 5,000 patients, have confirmed the benefits of EECP in providing symptomatic relief of angina (2, 7), improving blood flow to ischemic areas of myocardium (by radionuclide stress testing (6, 12) and positron emission tomography (PET) scans (11)), eliminating or reducing nitrate use (10), as well as improving exercise tolerance (1, 8). These effects have been reported to be sustained over several years (5, 9). However, despite the observed acute hemodynamic effects (systolic unloading, diastolic augmentation, and increased coronary blood flow) during EECP treatment and the neurohormonal changes (increased nitric oxide and decreased endothelin) associated with treatment, the mechanisms of action that can induce long-term improvement in the angina status of CAD patients remain unclear (4).

It has been proposed that the beneficial effects of EECP observed in clinical studies may be due to the formation of new blood vessels (angiogenesis), enhancement of collateral development from preexisting vessels (arteriogenesis), or an improvement in endovascular function (3). However, there is a relative paucity of basic scientific studies to support the proposed mechanisms. In addition, whereas EECP is usually administered for 35–36 h over 6 to 7 wk in patients with CAD, all previous animal studies have been acute experiments in which EECP was generally given for no more than 3 h. These studies are of limited value in extrapolating their findings to the effects of a prolonged course of EECP. The lack of an appropriate chronic EECP animal model has also limited research into EECP. The mechanism(s) of action whereby EECP produces sustained long-term benefits remains largely speculative.

We hypothesized that the beneficial long-term clinical effects of EECP may be related to the activation and expression of angiogenesis via the VEGF pathway as an initial process of microvessel development. The aim of this research was to determine in a simple myocardial infarction model whether chronic treatment with EECP has any angiogenic effects in the compression phase, increasing cardiac output by the Starling mechanism.

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infarct region of the myocardium. This model may not be useful in explaining the beneficial clinical results of EECP in the treatment of angina patients. On the other hand, the design of this model may be applicable to patients suffering from an acute or recent myocardial infarction. It is not known whether long-term EECP treatment would benefit this cohort of patients; and if so, is microvascular remodeling a possible mechanism of action? This model was therefore created to investigate this question.

MATERIALS AND METHODS

Animal preparation and catheter-based coronary occlusion model. This study was approved by the Animal Research Facility at Sun Yat-Sen University of Medical Sciences 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). Thirteen male beagle dogs ranging in age from 8 to 12 mo were included. For the development of myocardial infarction, animals were anesthetized with 3% pentobarbital sodium (1 mg/kg ip) injection and mechanically ventilated. The right femoral artery was accessed via the Seldinger technique, and an 8-Fr catheter was introduced to engage the left main coronary artery. An intracoronary occluder was deployed using the Seldinger technique, and an 0.014-in. intracoronary guide wire. The acute coronary occlusion was confirmed by typical ST-segment elevation on electrocardiography, specific cardiac enzymes, as well as cessation of antegrade flow at 30 min after coronary occlusion as assessed by angiography. Each dog was housed in a separate cage after the operation, and intensive care was given for the first 24 h. One dog died of ventricular arrhythmias on the third day after the operation. Therefore, a total of 12 beagle dogs survived the 1st and 2nd days and were randomized to the EECP group (n = 6 dogs, weight 14 ± 3.5 kg) or the control group (n = 6 dogs, weight 14.3 ± 2.6 kg).

EECP protocol and hemodynamic measurement in conscious dogs. A custom EECP device for conscious dogs mimicking the clinical patient treatment system was designed. It consisted of two pairs of pneumatic cuffs wrapped around the lower legs and thighs of the dogs. The cuffs were inflated at the end of systole from distal to proximal in a sequential manner and deflated at the end of diastole, in synchrony with the cardiac cycle. During the treatment, the dogs stood on the floor by their forelegs with their hindlegs suspended by adjustable belts to a support frame. The dogs were confined within the frame so as to minimize ECG interference due to animal movement. Each EECP dog received 1-h treatment daily for a total of 28 to 30 h over the 6-wk postocclusion period.

Long-term EECP has not previously been successfully performed in conscious animals. EECP was given 1 h per day, 5 days a week, based on the clinical EECP protocol for patients with CAD. During the EECP treatment, the dog was conscious with its lower body suspended by a metal frame. Control dogs were subjected to the same repeated instrumentation as the EECP dogs. However, actual treatment with active counterpulsation pressure was withheld in the control group.

The hemodynamic effects of EECP in conscious dogs were confirmed by invasive investigation conducted during the first EECP treatment. Each dog was anesthetized by an intraperitoneal injection of 3% pentobarbital sodium (1 ml/kg) and intubated for mechanical ventilation. The right carotid artery was cannulated by the Seldinger technique to allow for aortic pressure monitoring using a pressure transducer catheter (Millar Instrument), and the left carotid artery was exposed to measure blood flow using a 4-mm probe connected to an electromagnetic flowmeter (model MFV-2000). EECP was applied after the dog had completely recovered from anesthesia.

Assessment of myocardial perfusion and cardiac function. Four days and again 6 wk after LAD occlusion in both groups, myocardial perfusion was assessed by injecting 25 mCi 99mTc-labeled sestamibi (MIBI). Myocardial perfusion images were acquired using ECG-gated single-photon emission computed tomography (SPECT). Quantitative perfusion was analyzed by dividing the images into 20 segments in cross-sectional views at the apical and midventricular levels and a vertical long-axis view. The volumes of overall myocardium (Vt) and infarct-related myocardium (Vd) were calculated by tomographic reconstruction. A perfusion defect severity score (DS) was used to serve as an index of myocardial damage [DS = (Vt/Vd) × 100%]. Changes in perfusion in the infarcted regions were evaluated by comparing the DS at baseline to the DS at 6 wk.

Selective coronary angiography was performed before, at 30 min, and 6 wk after coronary occlusion. All animals were evaluated for global cardiac function [left ventricular ejection fraction (LVEF)] and regional wall motion (Cortina scores) by left ventriculography.

Tissue and serum sample collection. At the completion of the 6-wk experiment, Evans blue was injected through the root of the aorta by
clamping the ascending aorta. The infarction area was localized by Evans blue staining. All animals were euthanized by intravenous injection of a lethal dose of 10% potassium chloride, and their hearts were excised. Myocardial tissues were taken from the infarcted area of the left ventricle and a control region near the circumflex artery. Each tissue sample was divided into two sections. One section was fixed in 10% buffered formalin for immunostaining of microvessels and determination of VEGF expression, and another was immediately frozen in liquid nitrogen and then transferred to −80°C for VEGF mRNA expression by reverse transcriptase PCR (RT-PCR) analysis.

Peripheral blood samples were collected from all dogs at baseline, 6, 24, and 72 h after LAD occlusion and weekly thereafter. Serum was prepared and stored at −20°C for further determination of VEGF by ELISA kit (Chemicon, Temecula, CA) according to the manufacturer’s protocol.

**Histology and immunohistochemistry.** Formalin-fixed myocardial tissues from the infarcted region and from the control region in the proximal circumflex artery were embedded in paraffin and cut in 4-μm transmural sections for immunostaining of microvessels and VEGF expression. Sections were immunostained using the following antibodies: 1) monoclonal antibody against von Willebrand factor (vWF), a vascular endothelial cell marker (Dako, High Wycombe, UK); 2) polyclonal antibody against α-actin, a vascular smooth muscle cell marker (Dako); and 3) polyclonal antibody against VEGF (Oncogene, San Diego, CA) by using a modified avidin biotin-peroxidase protocol appropriate for each assay. In the control sections for each tissue sample, primary antisera was replaced with 1 mg/ml BSA (Sigma, Milwaukee, WI). Quantitative measurements of the positively stained areas were done from five random microscopic fields (×200) using computer-assisted morphometry. Positively stained microvessels were defined as round structures with a central lumen that were lined by a thin layer of endothelium that stained either vWF or α-actin. Microvessels showing positive staining for either vWF or α-actin were counted under a microscope with ×200 magnification. The number of microvessels identified from five fields in each section were averaged and expressed as “number per square millimeter of cross-sectional area.” Investigators performing histological and immunohistochemical analysis were blinded to animal treatment.

**VEGF mRNA expression by RT-PCR.** RT-PCR was conducted to detect the expression of VEGF mRNA. Total RNA was extracted from infarcted tissues that had been previously homogenized and prepared according to the Tri-Reagent protocol (Sigma, Milwaukee, WI). Total RNA was further fractionated in a 1.3% formaldehyde-agarose gel. Single-strand cDNA as a PCR template was synthesized using oligo(dT)12–18 primer and Moloney-murine leukemia virus reverse transcribe (Invitrogen, Carlsbad, CA). cDNA fragments of VEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified using Taq DNA polymerase. The sequences of the sense and antisense primers for VEGF and GAPDH were as follows: VEGF sense 5′-ATGACCTTTGCTTCTCGTGGT-3′, antisense 5′-CTTTGGTGCTCGATTACCATGGT-3′; GAPDH sense 5′-ATGTTCCAGTATGTTCTACCTTCTCAGTG-3′, antisense 5′-GCTTCACTACCTTTTCAGTGTCG-3′.

Conditions for the reaction were the following: 1× (94°C, 5 min), 25× (94°C, 1 min; 60°C, 1 min; 72°C, 1.5 min), and 1× (72°C, 7 min). Products were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide. The expression of GAPDH was referenced to an endogenous internal standard. Densitometry was applied to quantify VEGF mRNA expression in cardiac tissue.

**Statistical analysis.** Results of the quantitative studies were expressed as means ± SE. The unpaired Student’s t-test was used to compare the means. The number of stained vessels in infarcted myocardium was compared between groups using the Mann-Whitney nonparametric equivalence test. Statistical significance was accepted as P < 0.05.

Fig. 2. Myocardial perfusion restoration by EECP: A: representative images in each group demonstrate myocardial perfusion was significantly improved after 6 wk of EECP treatment (left) when compared with control (right). LAD, left anterior descending coronary artery. B: there was a significant difference in the change of defect score in the EECP-treated animals when compared with the control group (P < 0.05).
RESULTS

Hemodynamics in conscious dogs with coronary occlusion. Percutaneous acute coronary occlusion was successfully performed in all dogs and confirmed by selective coronary angiography, electrocardiography, and specific cardiac enzyme measurement (creatine kinase and creatine kinase-isoenzyme myocardial band). Central aortic pressure was recorded at the start of the first EECP treatment in the conscious dogs. Representative tracings of electrocardiography, aortic pressure, and carotid blood flow are shown in Fig. 1, demonstrating that EECP treatment indeed produced systolic unloading, diastolic augmentation, and increased carotid blood flow. The average peak resting aortic diastolic pressure was 127.3 ± 15.3 mmHg, measured at the closing of the aortic valve. This was significantly increased to 174.0 ± 18.4 mmHg during EECP (P < 0.01) and returned to 126.7 ± 17.2 mmHg 15 min after EECP. As shown in Fig. 1, the average peak systolic pressure at baseline was 159.7 ± 20.8 mmHg and was reduced to 140.5 ± 16.7 mmHg during EECP treatment, an average reduction of 19.2 ± 8.7 mmHg (P < 0.05). The ratio of peak diastolic pressure during EECP to systolic pressure was 1.24 ± 0.05. This is similar to the ratio obtained in the clinical setting.

Myocardial perfusion and cardiac function. Myocardial perfusion measured by ⁹⁹ᵐTc-MIBI SPECT significantly improved in five of six dogs in the EECP group. The mean DS pre-EECP was 19.6 ± 7.2% at baseline and 18.2 ± 6.8% post-EECP at 6 wk. In contrast, only one of the six dogs in the control group improved; the average DS at baseline of 19.1 ± 1.0% increased to 20.8 ± 1.9% at 6 wk. There was a significant difference in the change of DS in the EECP-treated animals versus the control group (P < 0.05) (Fig. 2, A and B).

At 30 min after LAD occlusion, LVEF decreased significantly from 65 ± 10.4% at baseline to 36.3 ± 6.6% (P < 0.01) in all animals, whereas the Cortina scores, indicating abnormal regional wall motion, increased dramatically from 6.1 ± 2.6% at baseline to 14.1 ± 5.3% (P < 0.05). After 6 wk, the LVEF in the EECP-treated group improved significantly from baseline (51.5 ± 14.9% vs. 36.3 ± 6.6%, P < 0.05) and the Cortina scores decreased from 14.1 ± 5.3% to 9.1 ± 2.8% (P < 0.05). In comparison, there were no significant changes at 6 wk in

A

\[\alpha\text{-actin immunostain} \quad \text{vWF immunostain}\]

Control

EECP

Fig. 3. Angiogenic response to EECP treatment in animals with myocardial infarction. A: there was more positive \(\alpha\text{-actin}\) and von Willebrand factor (vWF) staining in the infarcted myocardium of EECP dogs (bottom) in contrast to the control dogs (top) (\(\alpha\text{-actin} \times 200, \text{vWF} \times 400\)). B: bar graph demonstrates that the positive staining areas of both \(\alpha\text{-actin}\) and vWF in EECP animals were significantly increased compared with control group, respectively (*P < 0.05 when compared with the control).
either regional Cortina scores (13.8 ± 4.1% vs. 12.7 ± 3.8%) or global LVEF (35.33 ± 6.82% vs. 33.29 ± 5.12%) in the control group. These results demonstrate a significant improvement in global and regional function postmyocardial infarction after long-term EECP treatment.

**Microvessel development and angiogenesis.** In the EECP group, more microvessels developed in the infarct region after 6 wk of treatment, as assessed by immunohistochemical analysis with anti-vWF and anti-α-actin antibody labeling (Fig. 3A). The positive-stained area (in μm²) per sampling field by both anti-vWF and α-actin staining was significantly higher in the EECP group than in the control group: a threefold increase in anti-vWF antibody (5.6 × 10³ ± 1.8 × 10³ μm² vs. 1.7 × 10³ ± 1.5 × 10³ μm², P < 0.05); and a ninefold increase in α-actin (6.6 × 10³ ± 2.8 × 10³ μm² vs. 0.6 × 10³ ± 0.5 × 10³ μm², P < 0.05), as shown in Fig. 3B.

As an indicator of angiogenic response, EECP-treated animals showed a significantly greater number of positive vWF- and α-actin-stained microvessels in the infarcted regions than the untreated dogs. There were an average of 15.2 ± 6.3 microvessels stained positively with vWF per square millimeter of cross-sectional myocardial area versus 4.9 ± 2.1 microvessels in the control group (P < 0.05). Similarly, there were 11.8 ± 5.3 α-actin-stained microvessels in the EECP group versus 3.4 ± 1.2 microvessels in the control group (P < 0.05).

**VEGF response to long-term EECP treatment.** High VEGF expression was observed in the infarct zones of the EECP group after 6 wk. Generally, positive staining was predominantly limited to areas with residual cardiac myocytes. Interestingly, several other cell types also stained positively for VEGF in the EECP group, including vascular endothelial cells, fibroblasts, and macrophages. In contrast, fewer positively stained cells were found in controls (Fig. 4A). The positive-stained area per sampling field measured by computerized-assisted morphometry was a significant eightfold higher in the EECP group than in the control group (3.53 ± 1.11 μm² vs. 0.36 ± 0.28 μm², P < 0.01). The expression of VEGF mRNA in the cardiac tissue, determined by RT-PCR, showed a greater than 1.6-fold increase in VEGF mRNA in the EECP group compared with the controls (Fig. 4B).

The expression of VEGF mRNA in the cardiac tissue determined by RT-PCR showed no significant differences between infarct regions and noninfarct regions in the same heart. In areas of normal myocardium, expression of VEGF mRNA was also very weak, as shown in Fig. 5A. However, after long-term EECP treatment, there was a more than twofold increase in VEGF mRNA when compared with the controls (Fig. 5B). After acute coronary occlusion, serum VEGF levels of both groups began to rise immediately, reaching a peak level in 24 h. Circulating VEGF gradually decreased over time and then increased slightly in both groups. However, contrary to the expression of VEGF mRNA in the cardiac tissue, the pattern of serum VEGF levels in the EECP group was similar to that of the control group during the 6 wk. No significant difference was found between both groups in serum VEGF variation (Fig. 5C).

**DISCUSSION**

In the past decade, chronic animal studies of EECP have not been performed due to technical limitations, including animal
increasing coronary perfusion and salvaging chronically ischemic myocardium from necrosis, thus limiting infarct size, reducing consequent left ventricular dysfunction, and attenuating remodeling. The promotion of myocardial microvessel development was associated with the upregulated expression of VEGF in the infarcted myocardium as documented by immunohistochemical staining and RT-PCR analysis in the present study. Recently, a growing number of studies have demonstrated that mechanical stress, or shear stress, is one of the critical factors involved in the coronary angiogenesis. By effectively assisting the circulation, EECP noninvasively induces high pulsatile flows and increases shear stress on the endothelium, serving as a strong activator of VEGF. Interestingly, in the present study, VEGF local expression in the myocardium did not diminish with the improvement of myocardial ischemia; on the contrary, VEGF expression was locally enhanced in EECP dog. One explanation is that increase in blood flow produced by EECP treatment may serve as a strong stimulus for VEGF expression, even when myocardial ischemia was reduced. Increased VEGF expression may induce microvessel development and angiogenesis in the infarcted regions of the myocardium. In addition, pressure gradients across the coronary circulation (between epicardium and endocardium and between ischemic and nonischemic areas) produced by EECP during diastolic augmentation increase the coronary blood flow and shear stress and may stimulate angiogenesis. In the present study, a dose-response relation between EECP therapy and serum VEGF levels was not demonstrated. However, immunohistochemical staining and RT-PCR identified strong expression of VEGF in local infarcted myocardium in the EECP-treated group in contrast to the control group. One possible explanation is that EECP predominantly effects the coronary circulation by augmenting diastolic pulsatile flow and pressure, promoting conditions for VEGF production in the microcirculatory environment of the infarcted myocardium where myocytes may be in hibernation. 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 findings.

There are several limitations in this study. The myocardial infarction experimental model is not directly applicable to the chronic angina patients currently treated by EECP. Therefore, the results of this study may not serve as a mechanism of action to explain the beneficial effects observed clinically. However, EECP may be used in the treatment of patients with acute or recent myocardial infarction, and this paper may provide some evidence to justify this application. The study end point was chosen immediately after cessation of EECP to reduce contamination of the results with the time-dependent collateral development that is known to occur following a chronic occlusion. Whereas the choice of an early (6 wk postmyocardial infarction) end point more clearly identifies effects secondary to EECP, the long-term effects remain to be clarified.

Second, even though we have demonstrated an increase in newly formed microvessels in the infarcted regions of the myocardium after EECP, we did not measure an increase of absolute blood flow to these regions. The change in radionuclide perfusion is a relative measurement. In addition, the resolution of perfusion imaging is limited, and the changes in the defect scores we observed may be due to improved contractile function because of reduced systolic pressure and compliance. These limitations have hindered further study of EECP mechanisms. In the present study, we found the beagle dog to be an ideal candidate for chronic EECP because of its friendly and amenable temperament and excellent compliance. Alternative animal models such as swine are also both more difficult to train to stand still for EECP for an hour each day for 30 days and are temperamentally much more difficult to handle. Whereas the swine Ameroid ischemic model is perhaps more reflective of the chronic ischemia seen in patients with stable angina, it is difficult to quantify the degree of ischemia produced and would have required a much larger group of animals and financial support. The canine chronic occlusion model was chosen as being simpler, applying to the common human condition of postinfarct ischemia and requiring fewer experimental subjects.

This is the first report of chronic EECP treatment in conscious animals since EECP was developed in the early 1960s. Long-term EECP treatment may improve cardiac function by
therefore myocardial energy demand. However, because systolic pressure returned to baseline after EECP and there was a significant increase in microvessels in the infarcted regions, the improved defect score may indeed come from an increase in blood flow to these regions. Radionuclide perfusion (SPECT technetium defect score), global ejection fraction, and regional function (Cortina scores) all improved in the EECP-treated beagles in conjunction with the expression of growth factors and the development of microvessels. These findings suggest benefit for both myocardial functional restoration and remodeling postinfarction. The persistence of these findings will require further investigation. Finally, we did not perform quantitative histology to definitively define infarction regions and the existence of hibernating myocytes and to correlate these findings to improved defect scores and increased microvessels in these regions.

In summary, a chronic canine model of coronary occlusion was developed to investigate the effects of long-term EECP treatment on myocardial perfusion, cardiac function, as well as microvessel formation. Treatment with EECP provided effective diastolic augmentation and an increased myocardial perfusion pressure. EECP-treated dogs demonstrated significant long-term benefits in improving regional and global left ventricular function. An increase in microvessels and infarct zone VEGF levels was noted in the EECP-treated group.

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