PRECLINICAL RESEARCH

Erythropoietin Induces Neovascularization and Improves Cardiac Function in Rats With Heart Failure After Myocardial Infarction

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OBJECTIVES
We assessed the effects of erythropoietin (EPO) treatment in a rat model of post-myocardial infarction (MI) heart failure.

BACKGROUND
Erythropoietin, traditionally known as a hematopoietic hormone, has been linked to neovascularization. Whereas administration of EPO acutely after MI reduces infarct size and improves cardiac function, its role in the failing heart is unknown.

METHODS
Rats underwent coronary ligation or sham surgery. Rats with MI were randomly assigned to: untreated (MI), a single bolus of EPO immediately after MI induction (MI-EPO–early), EPO treatment immediately after MI and once every three weeks (MI-EPO–early + late), and EPO treatment starting three weeks after induction of MI, once every three weeks (MI-EPO–late). After nine weeks, hemodynamics, infarct size, myosin heavy chain (MHC) isoforms, myocyte hypertrophy, and capillary density were measured.

RESULTS
Erythropoietin treatment started immediately after MI (MI-EPO–early and MI-EPO–early + late) resulted in a 23% to 30% reduction in infarct size (p < 0.01) and, accordingly, hemodynamic improvement. Erythropoietin treatment, started three weeks after MI (MI-EPO–late), did not affect infarct size, but resulted in an improved cardiac performance, reflected by a 34% reduction in left ventricular end-diastolic pressure (p < 0.01), and 46% decrease in atrial natriuretic peptide levels (p < 0.05). The improved cardiac function was accompanied by an increased capillary density (p < 0.01), an increased capillary-to-myocyte ratio (p < 0.05), and a partial reversal of beta-MHC (p < 0.05) in all treated groups.

CONCLUSIONS
In addition to its effect on infarct size reduction, EPO treatment improves cardiac function in a rat model of post-MI heart failure. This observation may be explained by neovascularization, associated with an increased alpha-MHC expression. (J Am Coll Cardiol 2005;46:125–33) © 2005 by the American College of Cardiology Foundation

Erythropoietin (EPO) is best known as a hematopoietic growth factor, promoting proliferation and differentiation of erythroid progenitor cells. However, the expression of the EPO receptor outside the hematopoietic system, including endothelial cells, cardiomyocytes, and neurons, may suggest additional effects of EPO beyond hematopoiesis (1–4).

Because an insufficient amount of capillaries may lead to left ventricular (LV) dilation and heart failure after myocardial infarction (MI) (5), treatment directed towards increasing capillary density might be beneficial in heart failure. Expanding evidence shows that EPO is involved in angiogenesis. It has been shown that stimulation of cultured endothelial cells with EPO resulted in cell proliferation, chemotaxis, and differentiation into vascular structures (6). Furthermore, Jaquet et al. (7) found that EPO and vascular endothelial growth factor were equally effective in stimulating angiogenesis in endothelial cells derived from the myocardium. Most recently, it has been shown that EPO treatment in a rat stroke model resulted in an increased capillary density around the ischemic lesion (8).

In addition, EPO has been implicated to play a protective role during acute ischemia in brain (2,9,10) and heart (11–13). Pretreatment with exogenous EPO rescued hypoxic cultured cardiomyocytes from apoptosis (12); EPO perfusion during ex vivo ischemia-reperfusion improved LV function and reduced cellular damage (4,13,14). Acute, systemic treatment with EPO, in a rodent ischemia-reperfusion model, substantially reduced infarct size and decreased myocardial apoptosis (12), even when EPO was administered after reperfusion (11,15).

While the cardioprotective effects of EPO during acute MI are increasingly recognized, the role of EPO treatment in chronic heart failure (CHF) is unknown. Therefore, we assessed the effects of EPO treatment in a rat model of post-MI heart failure (16). In this model, induction of MI
leads to a time-related and infarct size-related ventricular dilatation and heart failure (17). We hypothesized that EPO treatment initiated after heart failure development (three weeks after induction of MI) would improve cardiac performance, possibly by increasing capillary density. To distinguish the acute effects of EPO (i.e., infarct size reduction) from its effects in CHF, we studied two additional groups. In one group we administered only a single dose of EPO immediately after MI, and in a second group we administered EPO immediately after MI and continued EPO treatment during the experiment.

METHODS

Animals. We used male Sprague Dawley rats weighing 270 to 330 g (Harlan, Zeist, the Netherlands). Animals were fed ad libitum, and housed in groups of four to five rats, according to institutional rules with 12:12 h light–dark cycles. The experimental protocol was approved by the Animal Ethical Committee of the University of Groningen.

Design of the study. Rats were either subjected to left coronary artery ligation (n = 85) or sham surgery (n = 8). Rats with MI were randomized to one of four groups; untreated (MI) or three different treatment strategies with EPO: a single bolus of EPO immediately after ligation (MI–EPO–early); EPO treatment directly after ligation and once every three weeks (MI–EPO–late); EPO treatment starting three weeks after ligation, once every three weeks (MI–EPO–early); and EPO treatment initiated after heart failure development (three weeks after induction of MI) would improve cardiac performance, possibly by increasing capillary density.

Infarct size and LV hypertrophy. After hemodynamic measurements, hearts were rapidly excised and weighed. Mid-papillary slices were prepared for immunohistochemistry. Slices were fixed in 4% paraformaldehyde and paraffin-embedded. Infarct size was determined by planimetry at mid-ventricular levels in transverse slices on picrosirius red/fast green-stained sections. Infarct size was expressed as the percentage of scar length to total LV circumference, as described previously (20,21). Deparaffinized 5-μm thick sections were stained with a Gomori’s silver staining, in order to visualize individual myocytes in the viable LV wall, the area with the most pronounced underperfusion (22). Using image analysis (Zeiss KS 400, Jena, Germany), concentric myocyte hypertrophy in the viable LV wall, remote from the infarcted area, was measured as the cross-sectional area of transversally cut myocytes showing a nucleus. Myocyte density was calculated as the average number of myocytes per tissue area. In each stained section, measurements were averaged from three different counting fields (±75 myocytes per heart).

Myosin heavy chain (MHC) isoform analysis. Samples of the LV (not infarct area), were frozen in liquid nitrogen and stored at −80°C. The freeze-dried samples were dissolved in a buffer, and gel electrophoresis was performed as described previously (23). Samples (0.5 μg) were run at constant current (24 mA) for 5 h. Silver staining of the gels and laser scanning densitometry was performed to identify differences in myosin isoform composition (i.e., alpha-MHC and beta-MHC).

Capillary density. To visualize capillaries in the myocardium in the same area as used for the measurements of the myocyte size, endothelial cells were stained with biont-labeled Lectin GSL (1:100; Sigma-Aldrich, St. Louis, Missouri), as previously described (16). Because lectins stain not only capillaries but also other vessels, a size criterion of

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<th>Abbreviations and Acronyms</th>
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<tr>
<td>CHF = chronic heart failure</td>
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<td>dLVP = developed left ventricular pressure</td>
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<td>EPO = erythropoietin</td>
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<td>LV = left ventricle/ventricular</td>
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<td>LVEDP = left ventricular end-diastolic pressure</td>
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<td>LVSP = left ventricular systolic pressure</td>
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<tr>
<td>MHC = myosin heavy chain</td>
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<td>MI = myocardial infarction</td>
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<td>N-ANP = N-terminal atrial natriuretic peptide</td>
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10 μm was used to exclude small arterioles and venules. Image analysis (Image Pro–plus version 4.5, Media Cybernetics Inc., Silver Spring, Maryland) was used to measure capillary density, calculated as the number of capillaries per tissue area. The measured total tissue area was corrected for the remaining interstitial space. Actual neovascularization was derived from an increased capillary-to-myocyte ratio, which has been calculated as capillary density divided by myocyte density (24).

**Statistical analysis.** Data are presented as mean ± SEM. Statistical analysis between groups was performed by one-way analysis of variance. When a statistically significant difference was detected, a Fisher protected LSD post-hoc analysis was performed. Correlation analysis was performed with Pearson’s correlation tests. Differences were considered significant at p < 0.05.

**RESULTS**

**General.** Overall mortality after MI was 41%. Mortality occurred only in the first 24 h after induction of MI. There were no statistically significant differences in mortality between the four groups (MI: 50%, MI-EPO–early: 40%, MI-EPO–late: 32%, and MI-EPO–early+late: 41%; p = 0.54). No mortality was observed in sham-operated rats. At baseline, no differences in body weight were observed (data not shown). General characteristics after nine weeks are shown in **Table 1.** Body weight was comparable among the five groups. Among groups with MI, systolic blood pressure was significantly lower only in MI and MI-EPO–early compared to sham; p < 0.01. Systolic blood pressure was significantly higher in MI-EPO–late and MI-EPO–early+late compared to MI group (p < 0.05). No significant differences were observed in heart rate and diastolic blood pressure, although there was a trend towards higher diastolic blood pressure in the groups repeatedly treated with EPO (MI-EPO–late and MI-EPO–early+late). The changes of the hematocrit throughout the experiment are also shown in **Table 1.** After nine weeks, hematocrit values were significantly elevated in the MI-EPO–late and MI-EPO–early+late compared to other groups.

**Infarct size.** Left ventricular infarct size (percent of LV) was comparable between MI and MI-EPO–late, 43% and 41%, respectively (p = 0.60; **Table 1**). Treatment with EPO immediately after coronary artery ligation reduced infarct size by 30% in MI-EPO–early and by 23% in MI-EPO–early+late groups (both p < 0.01 vs. MI; **Table 1**).

**Hemodynamic measurements.** Hemodynamic data obtained nine weeks after surgery are summarized in **Figure 1.** The LVSP and dLVP were both clearly diminished in MI compared to sham-operated rats (p < 0.01 for both); MI-EPO–late and MI-EPO–early+late showed a significantly higher LVSP and dLVP, compared to MI (all p < 0.05). One single bolus of EPO immediately after ligation (MI-EPO–early) did not result in a significantly improved LVSP or dLVP (Figs. 1A and 1B).

The LVEDP was elevated in MI compared to sham-operated rats (21 ± 3 mm Hg vs. 8 ± 1 mm Hg; p < 0.01). Importantly, EPO treatment started three weeks after MI (MI-EPO–late), resulted in a 34% decrease in LVEDP, compared to MI (p < 0.01), despite similar infarct sizes. Immediate treatment with EPO after induction of MI (MI-EPO–early and MI-EPO–early+late) led to a 27% and 38% reduction in LVEDP, respectively, compared to MI group (p < 0.05 and p < 0.01; **Fig. 1C**).

Myocardial contractility (−dP/dt\_max) and myocardial relaxation (−dP/dt\_max) were both impaired in MI compared to the sham group (both p < 0.01); MI-EPO–late and MI-EPO–early+late showed an improved contractility and relaxation compared to MI (all p < 0.05). In contrast, when only one single bolus of EPO was admin-

**Table 1.** Characteristics of the Experimental Groups

<table>
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<tr>
<th>General</th>
<th>Sham</th>
<th>MI</th>
<th>MI-EPO–Early</th>
<th>MI-EPO–Late</th>
<th>MI-EPO–Early+Late</th>
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<tr>
<td>Infarct size (% of LV)</td>
<td>8</td>
<td>12</td>
<td>12</td>
<td>13</td>
<td>13</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>313 ± 3</td>
<td>324 ± 6</td>
<td>332 ± 7</td>
<td>326 ± 7</td>
<td>328 ± 8</td>
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<tr>
<td>SBP (mm Hg)</td>
<td>127 ± 3</td>
<td>111 ± 4†</td>
<td>115 ± 3†</td>
<td>120 ± 3¶</td>
<td>122 ± 3¶</td>
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<td>DBP (mm Hg)</td>
<td>78 ± 2</td>
<td>78 ± 2</td>
<td>79 ± 2</td>
<td>83 ± 3</td>
<td>86 ± 2</td>
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<tr>
<td>Body/organ weight</td>
<td>390 ± 10</td>
<td>395 ± 11</td>
<td>401 ± 8</td>
<td>400 ± 7</td>
<td>421 ± 6</td>
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<td>BW (g)</td>
<td>3.9 ± 0.1</td>
<td>6.4 ± 1.0†</td>
<td>4.2 ± 0.5§</td>
<td>3.9 ± 0.1§</td>
<td>3.9 ± 0.2§</td>
</tr>
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<td>Lang weight/BW (mg/g)</td>
<td>3.2 ± 0.1</td>
<td>4.0 ± 0.2†</td>
<td>3.8 ± 0.1†</td>
<td>3.7 ± 0.1*</td>
<td>3.7 ± 0.1*</td>
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Data are presented as mean ± SEM. *p < 0.05; †p < 0.01 vs. sham; §p < 0.05; ¶p < 0.01 vs. MI.

BW = body weight; DBP = diastolic blood pressure; EPO = erythropoietin; LV = left ventricle; MI = myocardial infarction; n = number of animals; SBP = systolic blood pressure.
istered immediately after MI (MI-EPO–early), contractility and relaxation were not significantly improved compared to MI (Figs. 1D and 1E).

**N-terminal ANP levels.** Figure 2 shows that plasma N-ANP levels were three-fold increased in MI group (p < 0.01 vs. sham-operated animals). Furthermore, N-ANP levels were significantly reduced in the MI-EPO–late and MI-EPO–early groups (p < 0.05 and p < 0.01 vs. MI), returning to sham values (both p = NS vs. sham). The MI-EPO–early group showed a trend towards lower N-ANP levels (p = 0.07 vs. MI).

**Organ weights and LV hypertrophy.** As shown in Table 1, the ratios of heart weight to body weight and that of lung weight to body weight were significantly increased in the MI compared to the sham–operated group (both p < 0.01). Lung weight to body weight (an indirect expression of the LVEDP and, thus, severity of heart failure) was significantly reduced in all EPO treatment groups (all p < 0.01 vs. MI). A trend towards lower heart weight to body weight compared to MI was observed in MI-EPO–late and MI-EPO–early+late groups. Left ventricular hypertrophy was further studied by histological analysis. Representative photomicro-

![Figure 1](image1.png)

**Figure 1.** Effects of myocardial infarction (MI) and erythropoietin (EPO) treatment on hemodynamic parameters. dLVP = developed left ventricular pressure; LVEDP = left ventricular end-diastolic pressure; LVSP = left ventricular systolic pressure; dP/dt max = maximal rate of increase; dP/dt max = decrease of ventricular pressure. *p < 0.05 vs. MI; **p < 0.01 vs. MI; #p < 0.01 vs. sham.

![Figure 2](image2.png)

**Figure 2.** Plasma N-terminal atrial natriuretic peptide (N-terminal ANP) levels. EPO = erythropoietin. *p < 0.05 vs. myocardial infarction (MI); **p < 0.01 vs. MI; #p < 0.01 vs. sham.
graphs of Gomori-stained sections of the viable LV free wall are shown in Figure 3A. Myocardial infarction resulted in a 35% increase in myocyte cross-sectional area, compared to sham (p < 0.05). All EPO-treated groups showed a trend towards a smaller myocyte cross-sectional area, although this did not reach statistical significance (Fig. 3B).

**Differences in MHC isoform composition.** Relative proportion of cardiac alpha-MHC and beta-MHC were compared in LV protein samples between the five different groups. Myocardial infarction resulted in a more than five-fold increase in expression of beta-MHC, compared to sham-operated rats (p < 0.01); EPO treatment in all three groups reduced the expression of beta-MHC by 26% to 31%, compared to MI (p < 0.05; Fig. 4).

**Capillary density.** Capillaries stained with lectin were clearly discernable in the myocardium. Figure 5A shows representative photomicrographs of the five different groups. Capillary density was significantly reduced in the MI group compared to the sham group (p < 0.01); EPO treatment in all three groups prevented the decrease in capillary density after induction of MI and restored it to sham values, as shown in Figure 5B (p = NS vs. sham). Furthermore, in the MI-EPO–late and MI-EPO–early+late groups, we observed a 39% and 48% increase in capillary-to-myocyte ratio, respectively (p < 0.05 and p < 0.01 vs. MI), whereas MI-EPO–early showed a clear trend (p = 0.05 vs. MI) towards an increased capillary-to-myocyte ratio (Fig. 5C).

In order to relate LV functional parameters through an MHC shift to increased capillarization, correlations were determined. We observed a strong correlation between capillary density and beta-MHC expression (r = -0.47, p < 0.01) and subsequently between beta-MHC expression and cardiac contractility and relaxation, r = -0.52 and r = 0.61, respectively (both p < 0.01). Furthermore, capillary density was correlated with myocardial contractility (r = 0.32) and relaxation (r = -0.37; both p < 0.05).

**DISCUSSION**

In the present study, the effects of EPO treatment in a rat model of post-MI heart failure were examined. To our knowledge, this study shows for the first time that EPO treatment initiated three weeks after induction of MI results in an improved cardiac function, as shown by a 17% increase of dLVP at 34% reduction in LVEDP and a 46% decrease in N-ANP levels. Furthermore, our data indicate that EPO...
restores capillary density to sham levels and increases the capillary-to-myocyte ratio, indicating neovascularization.

**Myocardial structure and cardiac function.** Previous studies already revealed that EPO has ancillary properties besides hematopoiesis. One of the first studies on EPO in the heart showed that EPO injected intraperitoneally for seven days reduced cardiomyocyte loss by 50% after ischemia-reperfusion injury (25). These observations have been confirmed by others. Parsa et al. (12) showed a 25% reduction in infarct size after four days of permanent occlusion of the left circumflex coronary artery in rabbits. A single dose of EPO at the onset of MI reduced infarct size, which was accompanied by reductions in LV size and an improved LV ejection fraction, measured by echocardio-

![Figure 4](image4.png)

**Figure 4.** Effects of myocardial infarction (MI) and erythropoietin (EPO) treatment on beta-myosin heavy chain (MHC) protein expression as a percentage of total MHC expression. *p < 0.05 vs. MI; #p < 0.01 vs. sham.

![Image of tissue sections stained with lectin](image5a.png)

**Figure 5.** (A) Tissue sections stained with lectin in the viable free wall of the five different groups, showing individual capillaries. (B) Actual measurements for capillary density in number of capillaries per mm². (C) Bar graphs representing the capillary-to-myocyte ratio in the different treatment groups. EPO = erythropoietin. *p < 0.05 vs. myocardial infarction (MI); **p < 0.01 vs. MI; #p < 0.01 vs. sham.
vascular endothelial growth factor (7). In a rodent model of tissue, EPO stimulates capillary outgrowth comparable to EPO should be considered to elucidate the observed benefit explained by infarct size reduction, other properties of Because the effect of EPO treatment in this group could not in LVEDP, and restoring N-ANP levels to sham values. 

This is clearly demonstrated by the finding that EPO treatment, initiated three weeks after MI, although not significantly influence infarct size. Our results suggest a link between neovascularization and functional effects of EPO. The mechanism behind the effect of EPO on new blood vessel formation in the heart remains unknown. In general, stimulation of in situ endothelial cell proliferation or bone-marrow-derived endothelial progenitor cells might play a role. Previous work showed that EPO effectively increases the amount of circulating endothelial progenitor cells (32), and significantly induces angiogenesis (29). Future experiments are needed to delineate the mechanism of EPO-stimulated capillary growth.

Hematopoietic effect. Another important property of EPO that might be involved in the cardioprotective effect observed in our study is its hematopoietic effect. Human recombinant EPO increases the number of reticulocytes after administration to rats after 3 to 4 days with a maximum after 8 to 11 days (33). In our study, we observed significant hematocrit elevation one week after a single dose of EPO. In the groups treated with multiple EPO doses, hematocrit remained significantly elevated throughout the experiment. The beneficial effects seen in these groups might, thus, in part, be explained on the basis of increased oxygen-carrying capacity of blood. However, the effects of higher red blood cell mass on oxygen delivery is not straightforward, because elevated hematocrit may down-regulate nitric oxide synthesis and, thus, impair tissue blood flow (34). In the clinical setting, increasing the number of red blood cell mass by blood transfusion has been reported to improve outcome in elderly patients after acute MI (35). Nevertheless, this beneficial effect is only seen in patients with hematocrit <33%. On the other hand, reduction in the infarct size observed in the early treated groups could not be attributed to the hematopoietic effect of EPO, because cell death and MI expansion occur mainly during the first three days after ischemic insult (26) and, thus, before significant hematocrit elevation.

Conversely, an increase in hematocrit may itself tend to deteriorate myocardial perfusion through adverse rheological effects. Elevated hematocrit levels (up to 80%) in polycytemic mice, overexpressing EPO, enlarge cerebral infarct volumes and leukocyte infiltration after permanent occlusion of middle cerebral artery (36). Furthermore, EPO administration and consequent higher hematocrit has been associated with other adverse cardiovascular effects. Therapeutic levels of EPO may cause higher incidence of thrombosis (37) and could lead to blood pressure elevation (38). In the present study, rats repeatedly treated with EPO had a
higher systolic blood pressure. This increase might be related to the improved cardiac function; however, the systolic blood pressure remained below the values observed in the sham-operated group.

**Clinical implications.** In clinical settings, EPO treatment has already been used to correct anemia in patients with CHF. Anemia is frequently observed in patients with CHF and related to increased morbidity and mortality (39,40). Furthermore, not only anemia, but also elevated endogenous EPO levels, are independently associated with an impaired outcome in CHF (41). Normalization of hemoglobin levels in mildly anemic patients with CHF has a positive effect on LV ejection fraction (42) and peak VO₂ (43). In addition to correction of anemia, other nonhematopoietic effects of EPO may play a role in the improvement observed in patients with CHF treated with EPO.

Besides the treatment of anemia, EPO is currently under investigation for its neuroprotective properties. In the first clinical, randomized, proof-of-concept trial, EPO was given to patients with ischemic stroke (44); EPO administration in high doses (entire dose 100,000 IU given in three days) proved to be both safe and beneficial. Patients randomized to the EPO group showed significant improvement in clinical outcome parameters and a trend towards smaller infarct sizes.

However, chronic therapy with EPO is also associated with adverse effects related to hematocrit elevation, such as hypertension and thromboembolic complications. This could be overcome by using a lower dose of EPO, as shown by Bahlmann et al. (45). In this study, a low dose of darbepoetin (0.1 µg/kg/week) rendered tissue protection in the kidneys even without raising hematocrit levels. The recently discovered nonhematopoietic derivates of EPO retaining tissue protection but without the undesired effects on hematopoiesis may become another possibility for chronic administration (46).

**Study limitations.** Several limitations of the present study have to be acknowledged. Although a clear increase in capillary density and capillary-to-myocyte ratio was observed, the improvement of cardiac function might also be related to other effects of EPO treatment. Because we did not perform sequential measurements of cardiac function, further studies would be needed to specifically denote the time-dependent effect of EPO treatment on attenuation of heart failure development.

We did not measure the direct myocardial perfusion, and, therefore, functional evidence of an improved perfusion remains unclear. However, we observed a clear correlation between capillary density and beta-MHC expression and cardiac function. Furthermore, we used the Fisher LSD post-hoc statistical test for analyzing our data, which does not control for multiple comparisons.

**Conclusions.** In summary, the present study demonstrates that EPO treatment in a rat model of heart failure improves cardiac function beyond its effect on infarct size reduction. This improvement could be explained by the increased capillary density and capillary-to-myocyte ratio, indicating formation of new blood vessels.

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