Effects of Combination of Proliferative Agents and Erythropoietin on Left Ventricular Remodeling Post–Myocardial Infarction

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Abstract

Erythropoietin (EPO) has the potential to improve ischemic tissue by mobilizing endothelial progenitor cells and enhancing neovascularization. We hypothesized that combining EPO with human chorionic gonadotrophin (hCG) would improve post–myocardial infarction (MI) effects synergistically.

Methods: After MI, five to seven animals were randomly assigned to each of the following treatments: control; hCG; EPO; hCG + EPO, and prolactin (PRL) + EPO. Follow-up echocardiograms were performed to assess cardiac structure and function. Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay and western blot analysis for apoptosis-related proteins, and cell proliferation by immunostaining for Ki67 and c-kit cells.

Results: The MI-mediated increased chamber systolic dimension ($p < 0.05$ in controls) was attenuated by hCG, EPO, and hCG + EPO ($p < 0.05$ vs. control) but not PRL + EPO. Similarly all treatment groups, except PRL + EPO, reduced MI-induced increases ($p < 0.05$ vs. control) in ejection fraction (EF). The functional improvement in the EPO-treated groups was accompanied by increased capillary density. Apoptosis was markedly reduced in all treated groups. Significantly more cardiac c-kit$^+$ cells were found in the hCG + EPO group.

Conclusion: Our findings revealed that EPO, hCG, or their combination ameliorate cardiac remodeling post-MI. Whereas EPO stimulates neovascularization only and hCG + EPO stimulates c-kit$^+$ cell proliferation. These data suggest that combining mobilizing and proliferative agents adds to the durability and sustainability of cytokine-based therapies for remodeling post-MI. Clin Trans Sci 2011; Volume 4: 168–174

Keywords: cytokines, erythropoietin, myocardial infarction, heart failure, remodeling

Introduction

Cardiac failure, a disorder responsible for electrical instability and mechanical dysfunction of the heart, affects five million people in the United States. Despite current therapeutic strategies, morbidity and mortality remain high.[1] Recently, the use of cytokines and/or growth factors alone or in combination with cell therapy to treat myocardial infarction (MI) has been explored.[2–4] Erythropoietin (EPO), a cytokine produced by the kidney in response to hypoxia, regulates erythropoiesis and promotes the proliferation and differentiation of erythroid progenitor cells.[5] In addition, it has been reported that EPO is effective in protecting neural and myocardial tissues against ischemic injury, thereby expanding the biological role of EPO beyond hematopoiesis.[6–9] In recent studies, endothelial cells, fibroblasts, and a population of cardiomyocytes have been revealed to have the receptor for EPO (EPO-R).[10] Furthermore, other significant reported effects of EPO include prevention of apoptosis,[11] stimulation of myocyte proliferation in culture,[7] mobilization of progenitor cells,[8] and angiogenesis in some models.[9] We believe that EPO's role in erythropoetic differentiation may be essential in promoting cardiac regeneration.

Similar to EPO, increased levels of prolactin (PRL) are associated with a rise in the level of circulating erythroid precursor cells.[12] PRL receptor (PRL-R), which has been grouped into the family of the cytokine/hemopoietin receptors,[13] is also expressed on lymphoid and hemopoietic tissues.[14]

Recently, preclinical, experimental administration of human chorionic gonadotrophin (hCG), or epidermal growth factor, followed by EPO revealed proliferation of neural stem cells and maturation of newly formed neurons in a rodent stroke model.[6,15] Administration of hCG is believed to have a proliferative effect on neuronal cells and it is of interest to determine if there is a similar effect on cardiomyocytes. Here we test the hypothesis that the combination of hCG and EPO has a synergistic effect on ameliorating cardiac injury after MI. Our results show, surprisingly, that either agent alone or in combination exert similar effects on cardiac function; however, only the combination of agents led to the proliferation and/or recruitment of endogenous c-kit$^+$ cells suggesting that the combination therapy may enhance the durability of the therapeutic response.

Material and Methods

Animal model

All procedures in this study were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85–23, revised 1996) and were approved by the Johns Hopkins University and University of Miami Animal Care and Use Committee.

MI induced by coronary artery ligation was performed in male 6-month-old Wistar rats as described previously.[4] Animals were randomly assigned to one of five treatment groups:

Control ($n = 6$): 0.9% saline solution (of equal volume to hCG) administered by subcutaneous (SC) injection on days 1, 3, and 5 followed by administration of saline intravenously (IV) by Alzet® pump (Durect Corp., Cupertino, CA, USA) for 3 consecutive days beginning on day 7 after surgery.

(1) hCG ($n = 7$): hCG (440 IU/day) administered by SC injection on days 1, 3, and 5, followed by administration of saline IV by Alzet® pump for 3 consecutive days, beginning on day 7 after surgery.

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(2) EPO (n = 7): 0.9% saline solution (of equal volume to hCG) administered by SC injection on days 1, 3, and 5, followed by administration of EPO (1440 IU) IV by Alzet® pump for 3 consecutive days, beginning on day 7 after surgery.
(3) hCG + EPO (n = 5): hCG (440 IU/day) administered by SC injection on days 1, 3, and 5 followed by administration of EPO (1440 IU) IV by Alzet® pump for 3 consecutive days, beginning on day 7 after surgery.
(4) PRL + EPO (n = 7): PRL (400 µg/kg) administered SC for 5 consecutive days, beginning on day 1 after surgery and followed by administration of EPO (1440 IU) IV by Alzet® pump for 3 consecutive days, beginning on day 7 after surgery.

Echocardiography

Echocardiographic measurements were obtained at baseline, 24 hours, and 1, 2, 4, and 8 weeks following MI. Echocardiographic assessments were performed in anesthetized rats (2% isoflurane inhalation) using a Vevo-770 echocardiogram (Visual Sonics Inc., Toronto, ON, Canada) equipped with a 17.5-MHz transducer. Cardiac dimensions: Left ventricular (LV) end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and fractional shortening (FS) were recorded from M-mode images using averaged measurements from three to five consecutive cardiac cycles according to the American Society of Echocardiography.[16] Ejection fraction (EF) was calculated from bidimensional long-axis parasternal views taken through the infarcted area. All images were analyzed using Vevo 770 3.0.0 software (Visual Sonics Inc.).

Hematocrit

Hematocrit was assessed at baseline, prior to MI, and day 5, 11, and 21 after surgery.

Morphometric analysis

Rat hearts were processed using routine histological procedures. Five-micrometer sections were sliced and stained with hematoxylin/eosin (H&E) and Masson's trichrome. Five samples of H&E staining from each group were used to quantify capillaries. All measurements were performed on five high-power fields (20× magnification) of three different regions at midventricular level: border zone (BZ), infarct zone (IZ), and remote zone (RZ) using NIH ImageJ version 1.30v for Windows. A size criterion of 10 µm was used to exclude small arterioles and venules.

Myocardial infarct size was quantified by the circumferential extent of the scar and the percentage area fibrosis as previously described.[4]

Immunostaining and TUNEL assay

Immunostaining and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay were performed as previously described.[4] Briefly, paraffin sections were deparaffinized and rehydrated by immersion in xylene and a graded series of ethanols. Antigen retrieval was performed by a heat-induced method with citrate buffer (Dako, Carpinteria, CA, USA). After blocking with 10% normal donkey serum, sections were incubated with a primary antibody (see *Table S1*), at 37°C for 1 hour, followed by application of secondary antibody (Jackson Immunoresearch, West Grove, PA, USA). Omission of the primary antibodies on parallel sections was used as negative control. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA). The total numbers of positively stained cells were quantified per slide to calculate the number of cells per unit area (mm^2) on each sample. Morphometric analysis was performed by using Adobe Photoshop CS3 (San Jose, CA, USA).

To quantify apoptosis of cardiac cells, TUNEL assay was performed on paraffin-embedded tissue sections according to the manufacturer's protocol using a commercially available kit (*In Situ* Cell Death Detection Kit, POD, Roche Diagnostics GmbH, Germany). Slides were analyzed by fluorescent microscopy under 20× magnification. Apoptotic nuclei were identified by green fluorescence staining and expressed as a percentage per square millimeter (mm^2) from tissue sections per animal, distinguishing between BZ and RZ of the infarct. All images were obtained with fluorescent microscopy (Olympus IX81, Olympus America Inc., Center Valley, PA, USA) and/or a confocal laser scanning module (LSM 710, laser scanning microscope, Carl Zeiss Microimaging GmbH, Germany).

Western blot analysis

Proteins were quantified utilizing the Bradford quantification assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (30 µg) were resolved in 10% sodium dodecyl sulfate before (SDS) Page (Bis-Tris Gel; Invitrogen) and incubated overnight at 4°C with mouse anti-caspase-3 antibody (BD611048), mouse monoclonal antihuman proliferating cell nuclear antigen (PCNA) (sc25280), rabbit polyclonal antihuman Bax (sc493), and rabbit polyclonal antihuman Bcl2 (sc 492). The immunoblots were detected with femto by enhanced chemiluminescence reaction. Blots were semiquantified using Bio-Rad Quantity One® Image Quant software.

Drugs

PRL and hCG were provided by Dr. A. F. Parlow from NHHP (UCLA-Harbor, Torrance, CA, USA) and EPO (Epogen, Amgen Inc., Thousand Oaks, CA, USA) was purchased from Henry Schein.

Statistical analysis

All values are shown as mean ± standard error of mean (SEM). Echocardiographic parameters during 8-week follow-up were compared within and between groups using one-way analysis of variance (ANOVA) and two-way ANOVA for repeated measurements followed by *post hoc* tests, respectively. For a given parameter, $p < 0.05$ was considered significant. All tests were carried out using Sigma Stat 3.5 (Jandel, San Rafael, CA, USA).

Results

Impact on LV remodeling

Echocardiography showed similar baseline parameters and degrees of LV dilatation and decline in cardiac function in all groups after MI (*Table 1*). MI led to a time-dependent increase in LV chamber dimensions (*Figure 1A* and *B*). Treatment with hCG, EPO, or hCG + EPO, but not PRL + EPO, attenuated the MI-increased LVESD in comparison to control rats (*Figure 1B*). Similarly, the decline in FS was ameliorated with those treatments, except PRL + EPO (*Figure 1C*). Following MI, EF fell by approximately 40% at 24 hours. This reduction in LV function persisted throughout the 8-week period in control rats. In contrast, beginning at 2 weeks, treatment with either hCG, EPO, or hCG + EPO, but not PRL + EPO, significantly improved EF as compared with control rats (*Figure 1D*). Interestingly, although both hCG and EPO similarly improved EF, the combination of hCG and EPO was not synergistic in terms of ameliorating the decline in EF following MI.

Parameter	Control	hCG	EPO	hCG + EPO	PRL + EPO
LVEDD (mm)					
Bsl	8.0 ± 0.1	8.1 ± 0.1	7.8 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
24 h	8.4 ± 0.1	8.3 ± 0.4	7.6 ± 0.1	8.1 ± 0.2	8.1 ± 0.2
W8	9.7 ± 0.2*	9.6 ± 0.3*	9.2 ± 0.1*	9.9 ± 0.4*	9.9 ± 0.4*
LVESD (mm)					
Bsl	4.3 ± 0.2	4.5 ± 0.2	4.5 ± 0.1	4.2 ± 0.2	4.2 ± 0.2
24 h	5.6 ± 0.3	5.9 ± 0.5	5.7 ± 0.2	6.5 ± 0.4	6.7 ± 0.2
W8	8.1 ± 0.2*	7.1 ± 0.2*†	6.9 ± 0.2*†	7.3 ± 0.4*	8.2 ± 0.3*
FS (%)					
Bsl	49.4 ± 1.9	48.3 ± 2.2	47.6 ± 1.5	49.8 ± 1.2	49.2 ± 1.9
24 h	27.5 ± 2.0	26.4 ± 2.7	24.5 ± 2.1	21.0 ± 3.0	16.8 ± 1.7†
W8	19.9 ± 2.2*	25.2 ± 2.2*†	25.7 ± 2.5*†	23.7 ± 0.6*†	17.7 ± 1.6*
EF (%)					
Bsl	83.2 ± 1.4	80.3 ± 0.7	81.2 ± 1.2	80.5 ± 0.3	81.2 ± 0.9
24 h	46.5 ± 1.8	49.4 ± 2.5	48.3 ± 2.4	47.7 ± 1.8	46.6 ± 0.8
W8	46.7 ± 1.7*	57.4 ± 2.3*†	56.8 ± 3.6*†	54.6 ± 2.0*†	39.5 ± 2.8*

All values represent means ± SEM (n = 5–7). *$p < 0.05$ vs. baseline, same group; †$p < 0.05$ vs. control, same time point

Table 1. Echocardiographic measurements at baseline (Bsl), 24 h and 8 weeks (W8) after MI.

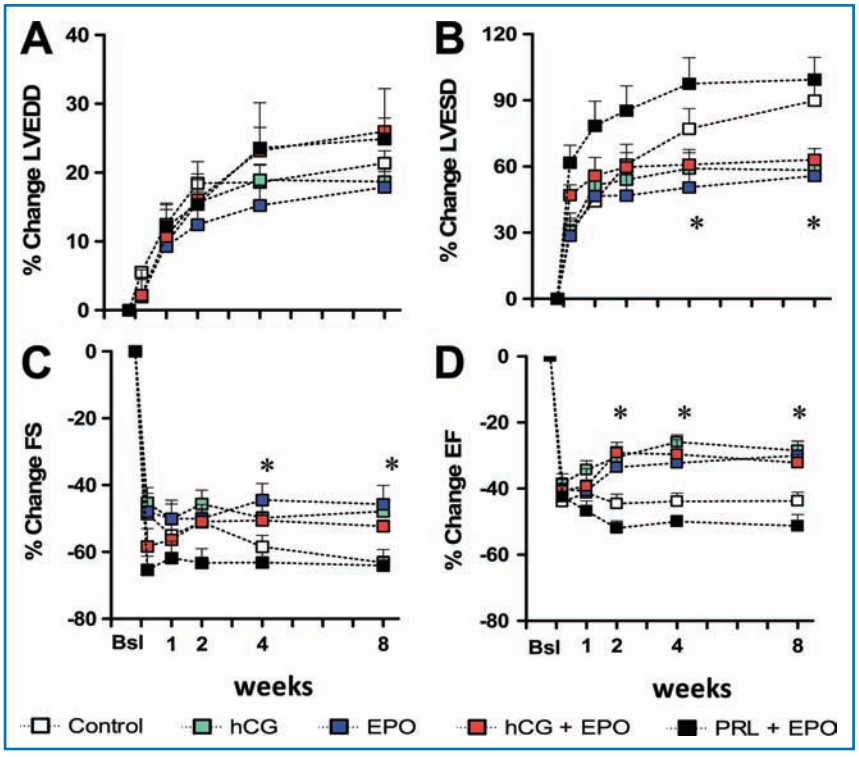

Figure 1. Impact of treatments on LV chamber size and function. Changes over time in (A) Left ventricular end-diastolic diameter (LVEDD); (B) Left ventricular end-systolic diameter (LVESD); (C) Fraction shortening (FS); and (D) Ejection fraction (EF). All values represent mean ± SEM. (*$p < 0.05$ for each treated group, except PRL + EPO, vs. control).

Impact on cellular division and proliferation

Next we sought to determine the cellular basis for the improved cardiac function after MI. Cellular proliferation assessed by Ki67 staining revealed that EPO and hCG + EPO (0.05 ± 0.003, 0.04 ± 0.003, cells per mm^2, respectively, $p < 0.05$ vs. control for each) suppressed proliferation in myocytes and nonmyocytes compared to control, whereas hCG alone or PRL + EPO (0.07 ± 0.002, 0.07 ± 0.003, cells per mm^2, respectively), did not affect cell proliferation (p = NS vs. control for each, *Figure 2A* and *B*, *Figure S1*). Moreover, the expression of PCNA as assessed by western blot analysis did not appear to change with the different treatments (*Figure S2*).

We next assessed endogenous c-kit$^+$ cardiac stem cell proliferation. Immunostaining revealed that with hCG or EPO alone there was no significant increased expression of c-kit$^+$ cells (0.0024 ± 0.0003/mm^2 and 0.0021 ± 0.0003, respectively). However, this combination (hCG + EPO) produced a three-fold increase in c-kit$^+$ cells (0.003 ± 0.0001 vs. 0.001 ± 0.0001, $p < 0.05$ vs. control, *Figure 3A* and *B*, *Figure S3*). Thus we concluded that the combination of agents is required for augmentation in the abundance of c-kit$^+$ stem cells.

Impact on cell death

TUNEL staining showed low levels of apoptosis in myocytes and nonmyocytes in all treated rats ($p < 0.01$ vs. control, *Figure 4A* and *Figure S1*). LV protein expression of caspase-3 was approximately 40% reduced in the hCG- and EPO-treated groups in comparison to control; however, neither of the combination groups (hCG + EPO and PRL + EPO) showed differences (*Figure S3*). In addition, the expression of Bax and Bcl2 was not different among groups (*Figure S3*).

Capillary density

Administration of EPO, alone or in combination with hCG resulted in a significant increase in capillary density, indicating new vessel formation or an angiogenetic effect. The increases with EPO alone or hCG + EPO occurred preferentially in the BZ and RZ, and did not occur directly within the IZ. EPO and hCG + EPO, but not hCG or PRL + EPO, increased overall capillary densities in BZ, IZ, and RZ by approximately 50% versus control as shown in *Figure 4B* and *Figure S4*.

Hematocrit measurements

While there was no change in the average hematocrit in rats treated with EPO compared to those receiving saline at either baseline, day 5, or day 11, the increased hematocrit in EPO-treated rats on day 21 was significant (*Table 2*).

Impact of treatments on myocardial infarct size

We also examined the effect of each treatment on scar size. Interestingly, scar size (*Figure 5A–F*) was only reduced in the group treated with hCG (*$p < 0.05$ vs. Control, †$p < 0.01$ vs. PRL + EPO).

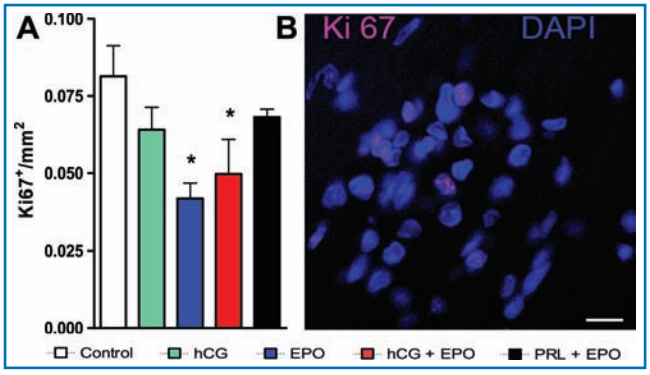

Figure 2. Impact of treatments on cell division. (A) Bar graphs showing the expression of cells positively stained for Ki67. All values represent mean ± SEM (*$p < 0.05$ vs. control). Bar graphs represent in white (control), green (hCG), blue (EPO), red (hCG + EPO), and black (PRL + EPO), respectively. (B) Representative confocal micrograph image of Ki67+ cells (magenta) and DAPI (blue) of the control group. Scale bar = 10 μm.

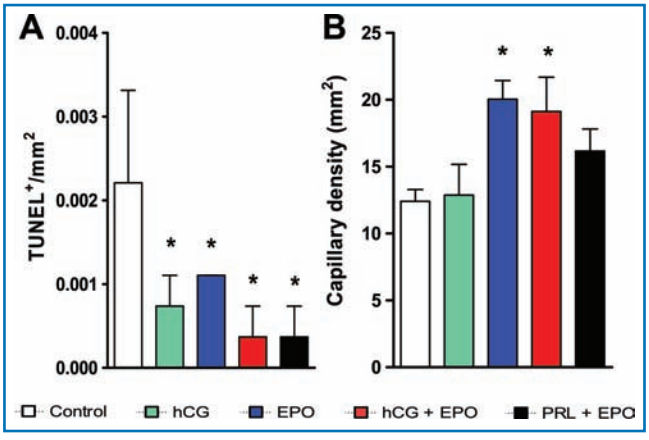

Figure 4. The antiapoptotic and angiogenic effects of treatments on the heart. All values represent mean ± SEM. (A) Bar graphs showing the expression of cells positively stained for TUNEL (*$p < 0.01$ vs. control). (B) Capillary density (*$p < 0.05$ vs. control). Bar graphs represent in white (control), green (hCG), blue (EPO), red (hCG + EPO), and black (PRL + EPO), respectively.

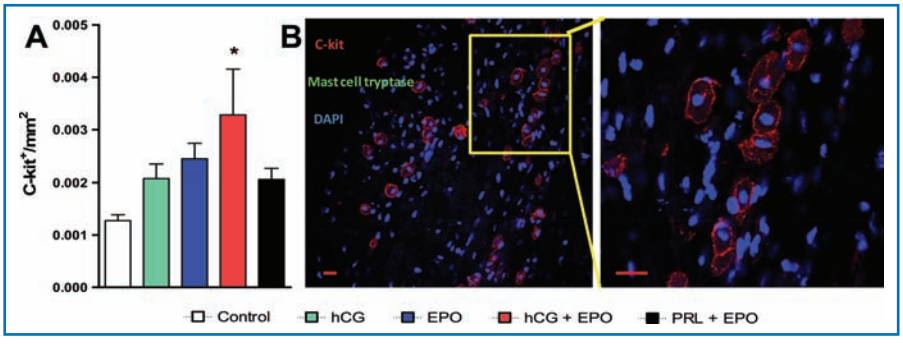

Figure 3. Impact of treatments on cardiac progenitor cells proliferation. All values represent mean ± SEM. (A) Bar graphs showing the expression of cells positively stained for c-kit (*$p < 0.05$ vs. control). Bar graphs represent in white (control), green (hCG), blue (EPO), red (hCG + EPO), and black (PRL + EPO), respectively. (B) Representative confocal micrograph image of c-kit+ cells (red), mast cell tryptase (green), and DAPI (blue) of the hCG + EPO-treated group. Scale bar = 10 μm.

Groups	Bsl	Day 5	Day 11	Day 21
Control	46.1 ± 0.6	42.6 ± 1.2	39.8 ± 1.3	43.4 ± 0.8
hCG	43.3 ± 0.6	41.4 ± 2.2	40.1 ± 1.2	45.3 ± 1.5
EPO	42.3 ± 0.9	41.7 ± 0.6	43.1 ± 1.4	52.6 ± 1.3*
hCG + EPO	44.0 ± 1.2	41.8 ± 0.9	42.2 ± 1.5	52.4 ± 1.7*
PRL + EPO	42.6 ± 0.6	41.6 ± 1.0	43.4 ± 1.0	47.6 ± 0.5†

All values represent means ± SEM ($n = 5–7$). (*$p < 0.001$, †$p < 0.05$ both vs. control).

Table 2. Serial measurements of hematocrit after treatment.

Discussion

The major new finding of this study is that EPO or hCG, either alone or in combination, ameliorates post-MI remodeling. This effect is associated with decreased apoptosis, increased capillary density, and c-kit+ cell proliferation. The latter occurs with greatest magnitude with the combination of these two drugs. These results complement those of earlier work, strongly suggesting that the administration of cytokines plays a role in the recruitment of stem cells into the region of injury.

Whereas, EPO was previously shown to potentially enhance cardiac repair after MI, the present findings are the first to document a cardiac reparative effect due to hCG, which is a glycoprotein hormone secreted by the placenta and not normally found in the nonpregnant circulation. hCG is structurally and functionally similar to luteinizing hormone (LH), which is primarily secreted by the anterior pituitary gland. Both hCG and LH bind the same receptor (LH/CG-R), which is a member of G-protein-coupled receptor (GPCR) family [17] and activates mainly the 3′-5′-cyclic adenosine monophosphate before (cAMP)/ protein kinase A (PKA) pathway.[18] In culture systems, hCG has been reported to promote survival and neurite outgrowth of primary neurons;[19] and Meng et al. [20] have demonstrated that hCG induces neuronal differentiation of PC12 cells through activation of LH/CG-R. Moreover, the expression of the LH/CG-R in the dentate gyrus of the hippocampus and the ependymal region, major sites known to harbor progenitor cells,[21] suggests the possibility that LH-induced LH/CG-R activation may be involved in neuronal renewal in the mature central nervous system before (CNS). Animal studies demonstrated that injection of hCG improved recovery of motor function in rats with complete spinal cord transection.[22,23] This phenomenon could be explained, at least in part, by neuronal regeneration from progenitor cells through LH/CG-R activation.

Interestingly, the effect of combination of hCG and EPO on the expression of c-kit+ cells in the heart was similar to the effects observed following activation of growth hormone releasing hormone before (GHRH) receptors as documented in our previous study.[4] The mechanisms underlying the overexpression of c-kit+ cells by treatment with these cytokines are still unclear, but because LH/CG-R and the receptor for GHRH are both members of GPCRs our findings suggest that both hCG and GHRH may share similar mechanisms for signal transduction after receptor activation in c-kit+ cells.

The improvement in cardiac function by EPO treatment observed in the present study is consistent with previous results.[24–27]

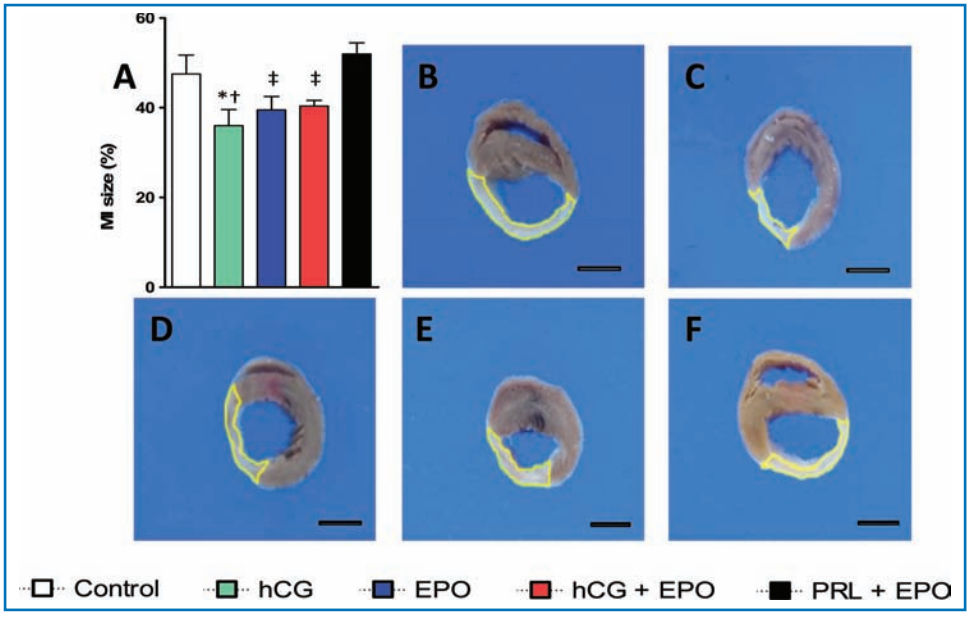

Figure 5. Impact of treatments on myocardial infarct size. Bar graphs (panel A) represent in white (control), green (hCG), blue (EPO), red (hCG + EPO), and black (PRL + EPO), respectively. All values represent mean ± SEM. (*$p < 0.05$ vs. control, †$p < 0.01$ vs. PRL + EPO, ‡$p < 0.05$ vs. PRL + EPO). Panels B, C, D, E, and F correspond to representative gross pathology images at midventricular level of control, hCG, EPO, hCG + EPO, and PRL and EPO, respectively. Scale bar = 5 mm.

Prunier et al. [26] showed hemodynamic improvement in maximum elastance in an MI group treated with higher dose of darbepoetin, an analogue of EPO. Moreover, Van Der et al.[24] demonstrated that EPO treatment improved cardiac hemodynamics in rats after MI, an effect that was accompanied by an increase in capillary density. Boucher et al.[28] showed that simultaneous administration of insulin-like growth factor-1 (IGF-1) and darbepoetin protects the rat myocardium against MI and enhances angiogenesis. An 8-week follow-up study accomplished by Moon et al.[25] showed that EPO treatment initiated 3 weeks after induction of MI resulted in an increase of LV pressure and reductions in LVEDP and LV size, and an improved LV EF as measured by echocardiography. Conversely, Hale et al.[29] demonstrated that treatment with EPO had no long-term beneficial effect on LV remodeling after MI, but may have exerted some positive effect on LV function. The main differences among the above studies may be explained by the treatment regimen. The most striking finding from the present study is that our regimen shows cardiac improvement both as early as 2 weeks and as late as 8 weeks, which has not been demonstrated by any regimen in previous studies.

Experimental efforts were devoted to reveal the potential mechanism(s) underlying the therapeutic effects of EPO and its combination with hCG and PRL by analyzing the degree of cellular apoptosis and proliferation. The administration of any treatment, except control, resulted in a significant reduction in the extent of apoptosis assessed by TUNEL assay. However, the extent of proliferation evaluated by Ki67 staining in our model was significantly inhibited by those treatments. This suggests that the therapeutic effect of EPO and hCG administration is partially due to a decrease in the extent of myocardial and nonmyocardial apoptosis, and not necessarily on the proliferation in the infarcted region in this model. This is consistent with previous studies in rats showing that a single treatment with EPO immediately or at different time points after release of coronary ligation, suppressed cardiac remodeling and functional deterioration, and decreased myocardial apoptosis.[30,31] Previous studies also showed that EPO stimulated proliferation or mobilization in some types of cells like endothelial progenitor cells.[8,32] In this experiment, the reduced proliferation level as revealed by Ki67 staining may be due to the fact that detection of Ki67 immunofluorescence only labels cells that were proliferating at the moment of euthanasia.[33] However, our subsequent experiments with c-kit$^+$ cells, histologically distinguished from mast cells, showed a trend to increase after treatment with each drug alone and a marked increase with the combination of hCG and EPO. Since the number of cardiomyocytes and noncardiomyocytes stained for Ki67 was generally reduced by these treatments, the increase in the c-kit$^+$ cells may indicate that the beneficial effects of these treatments are particularly associated with the proliferation of endothelial cells. In an effort to further reveal underlying mechanisms, we confirmed in the present study that EPO increases capillary density in infarcted hearts. Olivetti et al.[34] have provided experimental evidence showing that capillary numerical density within the uninjured tissue progressively decreased with infarct size leading to an increased diffusion distance for oxygen. Nishiya et al.[35] demonstrated enhancement of angiogenesis along with an increase in number of capillaries in EPO treatment groups. An increase in the number of capillaries in the BZ can improve oxygenation and nutrient supply after MI, prevent apoptosis, reduce progressive collagen deposition and scar formation, and improve ventricular function. Thus, neovascularization induced by EPO likely plays an important role in preventing cardiac remodeling. In a study conducted by Hirata et al.,[36] dogs exposed to EPO after coronary artery ligation showed mobilization of CD34$^+$ mononuclear cells and increased capillary density and myocardial blood flow in the ischemic region. Whether the increased capillary density by EPO treatment derives from mobilized stem cells such as endothelial progenitor cells remains unclear.

There are several potential mechanisms proposed to underlie EPO-mediated cardiac protection. Some suggested explanations include but are not limited to: (1) EPO protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production;[37] (2) EPO pretreatment can inhibit the activation of $NF_{\kappa}B$ and upregulation of TNF-α gene in cardiomyocytes exposed to hypoxia-reoxygenation injury through a negative feedback of $NF_{\kappa}B$-signaling pathway, and thus produces the antiinflammatory effect;[38] and (3) EPO may trigger cardiomyocyte proliferation and enhance phosphorylation of glycogen synthase kinase-3β.[39] Our results suggest that EPO has an early and long-term cardioprotective property that appears to be mediated by antiapoptotic and angiogenic effects. Importantly, despite the evidence of cardioprotection in experimental and clinical settings, more recent studies with conflicting efficacy results have raised safety concerns about EPO.[40] In a large clinical trial in stroke patients, the preliminary findings raised the concern of

potentially higher mortality.[41] However, while adverse effects were not observed during a 30-day follow-up, there was a trend toward a higher rate of adverse effects at 6 months on the Revival 3 trial (prospective, randomized, double-blind, placebo-controlled trial of erythropoietin in patients with ST-segment elevation myocardial infarction undergoing percutaneous coronary intervention). Thus, adverse effects such as increasing platelet activity, rise in blood pressure, and higher risk of thrombotic complications should be considered during long-term treatment.

To investigate the actual target of EPO, PRL was employed in the present study. Our results unexpectedly showed that the combination of PRL and EPO worsened the LV functional decline after MI. The inhibition of PRL on EPO-initiated therapeutic effect suggests a direct effect on the myocardium or activation/deactivation of specific pathways affected by EPO. Bellone et al.[12] reported that engagement of PRL with its receptor on a very early population of hemopoietic progenitors promotes the expression of EPO-R and thus the responsiveness to the progression factor EPO. The PRL-R and severely truncated EPO-R support differentiation of erythroid progenitors,[42] and the PRL-R rescues EPO-R$^{-/-}$ erythroid progenitors and replaces EPO-R in its synergistic interaction with c-kit.[43] Although there is little homology between the cytoplasmic domains of PRL-R and EPO-R, both receptors activate a very similar set of downstream signaling molecules, including JAK2 and STAT5, which are also activated by many other cytokine receptors,[44–46] including growth hormone receptor.[47,48] We noted that similar to PRL + EPO, treatment with rat recombinant growth hormone (rrGH) after MI worsened cardiac function and remodeling in previous studies.[4,49] Human GH also binds the murine PRL-R,[50] and some of the *in vivo* effects of rhGH may be due to activation of this pathway. Additionally, it is known that many of the growth-promoting effects of rhGH are mediated by IGF-1. IGF-1 stimulates rat PRL gene expression.[51] Studies have determined that *in vivo* administration of IGF-1 promotes hematopoiesis.[52] GH has also been shown to exert these effects[53] possibly in a manner similar to PRL. It is unclear if the effects of PRL on immunologic and hematopoietic development are direct or indirect. Overall, based on our findings we speculate that PRL as well as rrGH activate/deactivate similar remodeling pathways in the heart causing negative effects following MI, however, the mechanisms involved remain unclear.

Recently, it was suggested that hCG induces proliferation of neural stem cells and supports maturation of newly formed neurons in the rodent stroke model.[6,15] The purpose of the present study was to determine if hCG in combination with EPO provides any additional benefit in cardiac function than either one alone. Although the present data did not detect clear evidence of synergy between EPO and hCG in terms of functional recovery of the heart after infarction, the combination of agents led to stimulation of cardiac c-kit^{+} cells. Moreover, the data are the first to document amelioration of cardiac remodeling by hCG. Together these findings illustrate that activation of the LH/G-CR pathway in the heart is cardioprotective and may act by stimulating endogenous reparative pathways.

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Disclosures

Dr. Hare is a member of the Scientific Advisory Board of the Stem Cell Therapeutics Corp.

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