High-dose Erythropoietin Has No Long-term Protective Effects in Sheep with Reperfused Myocardial Infarction

Fernanda D. Olea, MSc, Gustavo Vera Janavel, MD,* Andrea De Lorenzi, MD,† Luis Cuniberti, PhD,‡ Gustavo Yannarelli, MSc,‡ Patricia Cabeza Meckert, MSc,†§ Martín Cearras, MD,* Rubén Laguens, MD, PhD,†‡ and Alberto Crottogini, MD, PhD**

Abstract: High-dose erythropoietin has been claimed to be cardioprotective in experimental acute myocardial infarction. In large mammals, however, results are controversial and long-term follow-up data are lacking. We thus assessed the long-term effects of high-dose erythropoietin on left ventricular infarct size and function in an ovine model of reperfused myocardial infarction. After 90 minutes of coronary occlusion followed by reperfusion, sheep received recombinant human erythropoietin (rhEPO) 3000 units/kg on 3 consecutive days (rhEPO group, n = 7) or vehicle (placebo group, n = 6). Ten weeks later, ventricular function was assessed by echocardiography and catheterization. Infarct size, evaluated as percent fibrotic myocardium (morphometry) and by hydroxyproline quantification, was similar in both groups (morphometry: rhEPO: 22.1 ± 5.5%, placebo: 18.1 ± 3.3%, *P* not significant; hydroxyproline: rhEPO: 6.6 ± 1.3 μg/mg wet weight, placebo: 7.1 ± 0.9 μg/mg, *P* not significant). Ventricular function was diminished in the rhEPO group, as indicated by lower septal wall thickening at the infarct border zone (rhEPO: −1.9 ± 16.4%, placebo: 20.5 ± 17%, $P < 0.04$), higher end systolic volume (rhEPO: 47 ± 14.3 mL, placebo: 32.6 ± 7.3 mL, $P < 0.05$), and higher end diastolic pressure (rhEPO: 17 ± 6.5 mm Hg, placebo: 10.1 ± 2.8 mm Hg, $P < 0.03$). In the rhEPO group, left ventricular endocardial area was larger, suggesting dilatation. High-dose erythropoietin has no cardioprotective effects in sheep with reperfused myocardial infarction.

Key Words: sheep, erythropoietin, myocardial infarction, reperfusion

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From the Departments of *Physiology; ‡Pathology, Favaloro University; †Institute of Cardiology and Cardiovascular Surgery, Favaloro Foundation, Buenos Aires; and §Scientific Investigation Commission (CIC), Buenos Aires Province, Argentina.
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Reprints: Alberto J. Crottogini, MD, PhD, Department of Physiology, Favaloro University, Solís 453 (1078) Buenos Aires, Argentina (e-mail: crottogini@favaloro.edu.ar).

In various species, erythropoietin (EPO) and its receptors have been detected in different nonhematopoietic tissues (brain, vascular system, heart, etc.),[1] suggesting additional effects of EPO beyond hematopoiesis.

EPO given in high doses has been shown to reduce cerebral infarct size and neuron apoptosis in rats[2] and to improve clinical outcome parameters in patients with ischemic stroke.[3] With regard to the heart, high-dose EPO has been studied in rodents with acute myocardial infarction (AMI)[4,5] or ischemia-reperfusion injury,[6–11] and in dogs with reperfused AMI.[12] These studies showed improved ventricular function and reduced infarct size. However, in pigs with reperfused AMI, EPO given before left anterior descending coronary artery (LAD) occlusion showed no infarct-limiting effect in the short term.[13]

Regarding the cardioprotective effect of high-dose EPO in longer follow-up periods, the results have also been controversial. Although Moon et al[14] and Van de Meer et al[15] showed reduced infarct size in rats at 8 or 9 weeks, respectively, after coronary artery ligation, Hale et al[16] found no such effect at 8 weeks post-AMI.

In addition to these controversies, long-term results in large mammals are lacking. This deficiency is important, especially considering that high-dose EPO has been proposed as a possible cardioprotective therapy in patients with AMI.[12] We therefore aimed to assess the effect of high-dose EPO on AMI size and left ventricular (LV) function in sheep with reperfused AMI at 10 weeks after LAD occlusion-reperfusion.

METHODS

Surgical Preparation and Experimental Design

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the Laboratory Animal Care and Use Committee of the Favaloro University. Thirteen Corriedale castrated male sheep were weighed and premedicated with intramuscular acepromazine maleate (0.2 mg/kg). Anesthesia was induced with intravenous (IV) sodium thiopental (20 mg/kg) and maintained with 3% halothane in pure oxygen under mechanical ventilation. During surgery and recovery, the electrocardiogram, heart rate, and oxygen

saturation (Novametrix 515A pulse oxymeter, Wallingford, CT) were monitored. After a sterile minithoracotomy at the fourth intercostal space, the LAD was occluded just before the emergence of the second diagonal branch for 90 minutes using a bulldog clamp. The infarcted zone was readily visible by the presence of cyanosis and dyskinesia. To reduce ventricular arrhythmias lidocaine (3 bolus injections of 2 mg/kg each every 20 min plus a 2 mg/kg infusion), amiodarone (150 mg in saline solution over 2 h), and atenolol (2 mg) were administered. At the onset of reperfusion, and 24 and 48 hours later, a 50-mL infusion containing recombinant human EPO (rhEPO, Hemax, Bio Sidus, Buenos Aires, Argentina) at a dose of 3000 units/kg (n = 7) or placebo (vehicle solution, consisting of human albumin 20% 2.5 mg/mL, sodium chloride 3.2 mg/mL, D-mannitol 25 mg/mL, sodium phosphate dibasic dodecahydrate 4 mg/mL, and sodium phosphate monobasic anhydrous 1.4 mg/mL; n = 6) was administered IV over 10 minutes. The thoracotomy was then closed and cephalotin (30 mg/kg IV) was injected. For all animals, survival time after AMI was 70 days. The nature of the infusates was kept blind for all investigators until the end of data collection and processing.

Serum rhEPO and Hematocrit

In all animals, hematocrit was measured before and at 11, 30, and 60 days after surgery. In 2 sheep of the treated group, rhEPO serum levels were determined with a Chemiluminescent Immunometric Assay (Immulite EPO, Diagnostic Products, Los Angeles, CA)[17] before and 30 minutes after each rhEPO infusion.

LV Function

On the day before killing, each animal underwent bidimensional echocardiography (Sonos 5500, Hewlett Packard, Boston, MA) under sedation with diazepam (10 mg IM). Percent systolic wall thickening at the infarct border zone of the anterior septum and in a zone remote to the LAD bed was measured as an indicator of regional function. The infarct border zone was considered to be the myocardium with some degree of motility lying closest to the akinetic area of the anterior septum. This zone was searched in each sheep individually, irrespective of any predetermined anatomic reference. Additionally, LV circumferential fractional shortening, LV end systolic volume, and LV end diastolic volume were determined as indexes of global LV function. It is to be noted that in the sheep, the distal LAD projects around the apex, the anterior two-thirds of the septum, and the junction of the right ventricular free wall with the anterior wall of the LV and its occlusion results in an antero-apical infarct.[18]

Hemodynamics

On the 70th day after surgery, the sheep were weighed and, immediately before killing, a pressure tip catheter (Millar MikroTip, Millar Instruments Inc., TX) previously calibrated (Xcaliber, Viggo-Spectramed, Oxnard, CA) was advanced via the left carotid artery into the LV chamber under sedation with sodium thiopental, to record the LV pressure signal (frequency: 250 Hz). The transducer control unit (TC-510, Millar) was connected to an amplifier (Gould Inc. 2400S, Cleveland, OH) and to a computer with the aid of an A/D expansion card.

LV peak systolic pressure, LV end diastolic pressure, and the peak rates of LV pressure increase and decay (dP/dt_{max} and dP/dt_{min}, respectively) were calculated using software developed in our laboratory. For each variable, we averaged the values of all beats recorded during a fixed 5-second acquisition time. Cardiac output (Siemens Sirecust 404-1 Thermodilution Cardiac Output Computer, Berlin, Germany) was measured by introducing a Swan-Ganz catheter in the left jugular vein and advancing it to the wedge position. The value for cardiac output (calculated from the average of 3 to 4 measurements) was divided by the body surface area to obtain the cardiac index. Immediately after cardiac output measurement, sheep were killed with an overdose of sodium thiopental followed by a bolus injection of potassium chloride.

Infarct Size

The heart was excised and the atria and right ventricle were discarded. The LV was weighed and cut open parallel to the posterior interventricular groove. Digital photographs of the endocardial surface were obtained for image processing (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD) to calculate total LV area but not infarct size, due to the unclear demarcation of reperfused infarct limits. The LV was cut in 8 longitudinal sections extended from the mitral valve annulus to the apex. Each section was further divided longitudinally into 2 sections to measure infarct size by 2 different means: one by morphometric assessment of fibrosis (these 8 pieces were fixed by immersion in 10% formaldehyde) and the other for determination of collagen content by hydroxyproline (Hyp) quantification (these 8 pieces were frozen at $-80^{\circ}C$).

For the morphometric study, each of the 8 formaldehyde-fixed pieces was fractioned into 4 segments and embedded in paraffin, maintaining the longitudinal orientation of the sections. In this way, 32 blocks for each heart were obtained. From each block, serial 4-μm thick slices extending from the epicardium to the endocardium were cut and stained with Masson trichrome. The proportion of myocytes and blue-stained collagen respective to the whole area was determined by digital analysis in each tissue section at 4× magnification in high-resolution scanned digital images. Given that we did not quantify the area at risk, the morphometrically studied infarct size is expressed as percent total LV area.

For Hyp quantification, each piece was thawed, weighed, and pulverized in liquid nitrogen. The powder was placed in 10 mL glass ampoules containing 4 mL of HCl 6 M and hydrolyzed at 110°C overnight. The content was filtered and centrifuged at 3000 rpm for 10 minutes. The supernatant was kept at $-20^{\circ}C$ and neutralized with

NaOH solution to achieve the pH of HCl 1 mM, for later photocolorimetric determination of Hyp concentration according to the Neuman and Logan method.[19] Each sample was prepared in duplicate and measured at 560 nm (CL-770 Clinical Spectrophotometer, Shimazu Co., Japan). The concentration of Hyp was obtained from a calibration curve and expressed as μg Hyp/mg wet weight of myocardial tissue. Finally, the Hyp concentration for each heart was calculated as the average of the 8 individual sample results.

Statistics

Placebo and rhEPO group results were compared using an unpaired Student *t* test. Data for defibrillation were compared using a Fisher exact test. Results are expressed as mean ± SD. Statistical significance was set at $P < 0.05$.

RESULTS

Body weight at baseline was 29.7 ± 3 kg for the EPO group and 28.2 ± 2.7 kg for the placebo group [*P* not significant (NS)] and at the end of the study, 35.1 ± 2.7 kg for the EPO group and 35.3 ± 2.8 kg for the placebo group (*P* = NS). Heart rate did not differ between groups either before (rhEPO group: 85.6 ± 10.8, placebo group: 80.8 ± 11.3, *P* = NS) or after (rhEPO group: 85.7 ± 10.8, placebo group: 80.7 ± 12.4, *P* = NS) LAD ligation. Oxygen saturation was also similar in both groups before (rhEPO group: 97.8 ± 2.8%, placebo group: 99 ± 0.8%, *P* = NS) and after LAD ligation (rhEPO group: 99 ± 1.4%, placebo group: 99 ± 1%, *P* = NS). Despite the use of antiarrhythmic drugs, ventricular arrhythmias were frequent, both during ischemia and reperfusion. Intraoperative defibrillation was carried out in 3 (42.9%) out of 7 rhEPO-treated sheep, and in 2 (33.3%) of 6 sheep receiving placebo (*P* = NS). In all cases, defibrillation was successful.

Hematocrit and Serum rhEPO

At 11 days after surgery, hematocrit was higher in the rhEPO-treated group (39.3 ± 2.3% vs. 36.3 ± 0.8%, $P < 0.02$). At the other 3 time points (0, 30, and 60 days), no differences were found. In the 2 sheep where serum rhEPO was assessed, it was undetectable before treatment and reached a maximum of 126.1 units/mL in one sheep and 109.3 units/mL in the other after the final infusion.

LV Function and Hemodynamics

Table 1 shows echocardiographic and hemodynamic data in rhEPO-treated and placebo-treated groups at 10 weeks after treatment. The anterior septum was dyskinetic in the rhEPO-treated group and only mildly hypokinetic in the placebo group, the differences being significant. In contrast, no differences in % wall thickening were observed in the noninfarcted LV lateral wall. The values for LV volumes were higher in the rhEPO-treated group, yet only in the case of end systolic volume did the difference achieve statistical significance. In the rhEPO-treated group LV end diastolic pressure was significantly higher. No differences between groups were found in the remaining parameters.

TABLE 1. Left Ventricular Function and Hemodynamics

	Placebo Group	rhEPO Group
Echocardiography		
% LVWTh (infarct border)	20.5 ± 17	− 1.9 ± 16.4*
% LVWTh (noninfarcted zone)	38 ± 15.7	32.6 ± 15.9
% LVFS	29.2 ± 12.3	32.8 ± 16.2
LVEDV (mL)	65.4 ± 9.4	82.8 ± 23.3
LVESV (mL)	32.6 ± 7.3	47 ± 14.3*
% EF	49.8 ± 10.9	42.6 ± 11.3
Left catheterisation		
LVPSP (mm Hg)	114.9 ± 14.5	116.7 ± 17.7
LVEDP (mm Hg)	10.1 ± 2.8	17 ± 6.5**
dP/dt_{max} (mm Hg/s)	2376.1 ± 689.2	2556.8 ± 1721.4
dP/dt_{min} (mm Hg/s)	− 2430.1 ± 545	− 2202.6 ± 597.8
HR (beats/min)	127.8 ± 19.2	111.7 ± 26.6
Right catheterisation		
CO (L/min)	5.1 ± 0.7	4.8 ± 0.8
CI (L/min/m^2)	5.5 ± 0.8	5.3 ± 1

CI indicates cardiac index; CO, cardiac output; dP/dt_{max} and dP/dt_{min}, peak rate of left ventricular pressure increase and decay, respectively; EF, ejection fraction; HR, heart rate; LVEDP, left ventricular end diastolic pressure; LVEDV, left ventricular end diastolic volume; LVESV, left ventricular end systolic volume; LVFS, left ventricular fractional shortening; LVPSP, left ventricular peak systolic pressure; LVWTh, left ventricular septal wall thickening.

Data are expressed as mean ± SD; n = 7 (rhEPO group) and n = 6 (placebo group).

Significant differences from the placebo group by Student *t* test are indicated with *$P < 0.05$ or **$P < 0.03$.

Infarct Size

Total LV endocardial area was larger in the rhEPO-treated group (83.2 ± 2.5 cm^2) than in the placebo group (76.9 ± 3.6 cm^2, $P < 0.01$). Percent fibrosis, as measured from tissue sections (Fig. 1), was similar in both groups (rhEPO: 22.1 ± 5.5%, placebo: 18.1 ± 3.3%, *P* = NS). The same occurred with Hyp content (rhEPO: 6.6 ± 1.3 μg/mg wet weight, placebo: 7.1 ± 0.9 μg/mg, *P* = NS). These results indicate that there were no

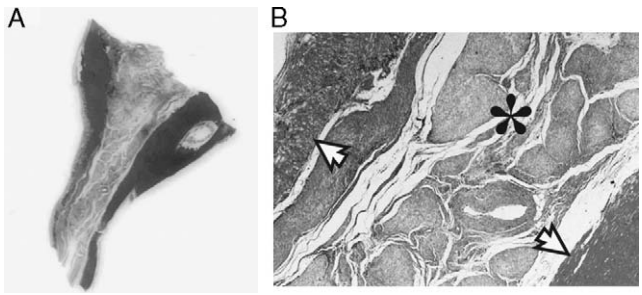

FIGURE 1. Morphometric analysis of infarct size. A, One of the tissue sections used for the morphometric analysis, stained with Masson trichrome (collagen light grey, myocytes dark grey), scanned at 1× and analyzed at 4×. At higher magnification (B, 40×) the collagen scar (asterisk) is surrounded by myocardium both at the endocardial and epicardial sides (arrowheads).

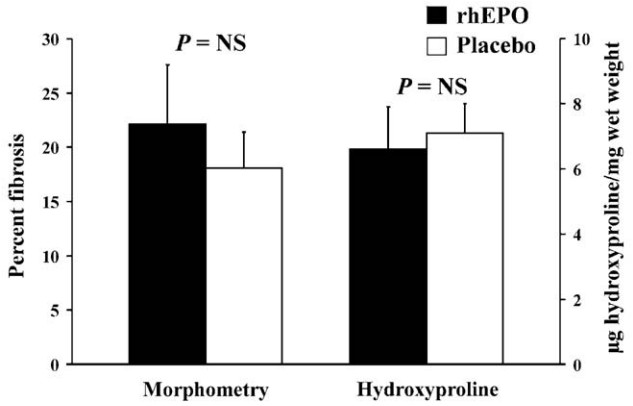

FIGURE 2. Infarct size calculated as percent fibrosis in paraffin-embedded tissue sections (morphometry) and as collagen content in myocardial tissue samples (hydroxyproline quantification). The size of the infarcts did not differ between groups with either method. n = 7 (rhEPO group) and n = 6 (placebo group). Data are expressed as mean ± SD.

differences in the extent of infarcted area between groups (Fig. 2).

DISCUSSION

To our knowledge, the present study is the first to report the long-term effects of high-dose rhEPO in a large mammalian model of reperfused AMI.

Our results show that in sheep undergoing 90 minutes of acute LAD occlusion followed by reperfusion, the daily infusion of 3000 units/kg rhEPO for 3 days does not reduce infarct size at 10 weeks after AMI. Moreover, some parameters of LV performance, such as wall thickening at the infarct border, LV end systolic volume, and LV end diastolic pressure showed functional impairment in the treated group. In addition, the rhEPO treated group exhibited enlarged LV endocardial area in the context of similar baseline heart sizes, suggesting dilatation and hence some degree of ventricular remodeling.

Most studies using high-dose rhEPO in AMI have been carried out in rodents and have analyzed short-term outcomes,[4–11] demonstrating cardioprotective effects of rhEPO through the activation of multiple antiapoptotic signaling pathways.[5,20,21] The few studies performed on large mammalian models of reperfused AMI have analyzed the short-term outcome (2.5 and 6 h) and have yielded controversial results.[12,13] Although in dogs, Hirata et al[12] showed reduced infarct size when administering EPO before reperfusion, in pigs, Kristensen et al[13] did not observe any beneficial effects of EPO given before coronary occlusion.

On the basis of reports on small rodents, where single or repetitive doses of 3000 to 5000 units/kg of rhEPO reduced infarct size and improved LV function in AMI with and without reperfusion, we used 3000 units/kg/d for 3 days and failed to observe cardioprotection.

Longer follow-up periods have been evaluated in rats and the results have also been controversial. In a rat model of AMI treated with EPO (5000 units/kg/d for 7 d), Hale et al[16] observed no infarct size reduction at 6 weeks. They speculate that this could be due to the fact that their model did not include reperfusion, a condition that exhibits high apoptotic activity in cardiomyocytes. Our experimental model did include reperfusion and even so we failed to show benefits from rhEPO. Hale et al also attribute their negative results to an excessive increase in hematocrit (67 ± 1%), which could cause hypertension and thrombotic events. In our case, although the hematocrit was significantly higher in the rhEPO-treated group at 11 days, the increase was transient and it did not exceed normal limits. The difference in hematocrit between groups indicates that rhEPO indeed had an effect in the sheep, ruling out the possibility that our negative results could be due to a lack of recognition of rhEPO by this species.

Our rhEPO-treated sheep showed some evidence of impaired LV function, which cannot be explained with the present data. Some studies have shown that rhEPO increases the platelet count, coagulation factor VIII, von Willebrand factor, and thrombin,[22] and reduces coagulation cascade inhibitors such as protein C and S, thus encouraging a prothrombotic state.[23] In effect, a reduction of bleeding time has been observed to occur even before the rise in hematocrit,[22,24] suggesting increased platelet activity. However, we did not find any histologic evidence of microthrombi or microinfarcts, suggesting that the thrombotic complications of rhEPO did not play a role in LV function impairment.

Severe hypertension is another adverse effect of EPO not only in patients but also in experimental animals.[25] Although at the end of the study, LV systolic pressure was similar in both groups, we cannot rule out that hypertension may have occurred at earlier times, thus causing pressure overload to the LV. In any case, it should be noted that our aim was to investigate the effects of high-dose rhEPO in this setting but not the mechanisms involved in the effects.

A comment should be made on our experimental model. The sheep is known to have no innate collaterals,[18] thus implying that coronary occlusion would result in homogeneous damage on all cardiomyocytes supplied by that artery. After 90 minutes of coronary occlusion, the question remains on how much of the area at risk is recoverable. In sheep with 90 minutes of LAD occlusion, Ko et al[26] showed that at 6 hours of reperfusion 75 ± 10% of the LV mass at risk undergoes infarction. Although further data are lacking, it is reasonable to assume that part of the cell death process occurs after releasing the occlusion, as a result of reperfusion injury. It is therefore sound to speculate that the effect of our rhEPO infusion (if any) given at the onset of reperfusion, would be to protect this latter mass from

infarction. In addition, given that the cell death process continues beyond 6 hours of reperfusion, the extra rhEPO administered at 24 and 48 hours would salvage at least part of the 25% area at risk that otherwise would have evolved into infarction. We thus believe that the lack of infarct-limiting effect of rhEPO is not due to the nature of the model used.

Central body temperature at the time of ischemia exerts a significant influence on infarct size. As premedication and anesthesia interfere with thermoregulation, body temperature may have varied heterogeneously among animals. We did not monitor this parameter, but the randomized nature of the study allows assuming that any bias on infarct size was evenly distributed between groups.

Finally, we cannot rule out that the antiarrhythmic drugs used in our sheep may have interfered with some potential effects of rhEPO on the myocardium in which ATP-dependent K^+ channels are involved.[27] As amiodarone and lidocaine have been shown to inhibit these channels,[28,29] the possibility exists that mitochondrial cell-death pathways, such as permeability transition pore formation and proapoptotic factors release, may have been operative.[30]

CONCLUSION

In sheep with reperfused myocardial infarction, rhEPO given in high doses early after coronary occlusion, does not reduce infarct size and apparently induces an impairment of LV function at 10 weeks after AMI. These results suggest that before high-dose EPO can be considered as a cardioprotective agent in patients subjected to reperfusion treatments after AMI, further studies are warranted.

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