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Combined VEGF gene transfer and erythropoietin in ovine reperfused myocardial infarction ☆

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ABSTRACT

Background: In reperfused acute myocardial infarction (RAMI), cardioprotective treatments may enhance myocardial salvage and hence reduce the area of necrosis. Based on studies showing that plasmid-mediated vascular endothelial growth factor (pVEGF) gene transfer reduces infarct size by combining angio-arteriogenic and cardiomyogenic effects and that erythropoietin (EPO) exerts anti-apoptotic actions in animal models of AMI, we aimed to assess if their association would reduce infarct size to a larger extent than any of them individually in a large mammalian model of RAMI.

Methods: Adult sheep subjected to 90-minute coronary artery occlusion received upon reperfusion intramyocardial pVEGF 3.8 mg plus intravenous EPO 1000 IU/kg (n = 8), pVEGF (n = 8), EPO (n = 8) or placebo (n = 8).

Results: Fifteen days after treatment, infarct size was smaller in the 3 treatment groups (pVEGF + EPO: 8 ± 1%; pVEGF: 16 ± 5%; EPO: 13 ± 4%) compared to placebo (25 ± 7%, $p < 0.001$). However, in the EPO + VEGF group infarct size was significantly smaller than in the groups receiving EPO or VEGF individually ($p < 0.05$). DNA fragmentation, a hallmark of late apoptosis, was significantly lower in sheep receiving EPO. The combined treatment, while not affecting global left ventricular performance, improved regional peri-infarct function and prevented over-time expansion of the post-infarct perfusion defect.

Conclusions: Combined pVEGF and EPO treatment might be clinically useful to enhance the benefits of early revascularization in patients with acute myocardial infarction.

1. Introduction

In the context of acute myocardial infarction (AMI), early reestablishment of coronary blood flow is currently the most useful tool to limit the necrotic area [1,2]. However, since the extent of the remaining fibrotic scar is directly associated with the severity of the ensuing left ventricular (LV) remodeling process that leads to heart failure [3], strategies directed to further reduce the size of reperfused AMI are of clinical interest.

In an ovine model of permanent coronary occlusion, we have shown that direct intramyocardial injection of a plasmid encoding human $VEGF_{165}$ (pVEGF) reduces infarct size by combining angiogenic, cardiomyogenic and antifibrotic effects [4].

On the other hand, high-dose erythropoietin (EPO) has been shown to exert antiapoptotic effects in isolated cardiomyocytes [5], ex-vivo hearts [6] and animal models of AMI [7–11]. While other mechanisms of EPO-induced cardioprotection, such as angiogenesis, appear to be mediated by VEGF transcription [12], inhibition of ischemia-induced apoptosis occurs through an Akt-dependent pathway [8,9,13].

It is therefore reasonable to hypothesize that EPO would act synergistically with VEGF against ischemic damage by contributing its anti-apoptotic action. Accordingly, in the present study we aimed to assess the effect of combined intravenous EPO and plasmid-mediated intramyocardial $VEGF_{165}$ gene transfer on infarct size, myocardial perfusion and left ventricular function in an ovine model of reperfused AMI.

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2. Materials and methods

2.1. Plasmid construct

The eukaryotic expression vector (pVEGF, deposited as pBSVEK3 at *Deutsche Sammlung von Mikroorganismen und Zellkulturen*, accession number DSM14346) is a 3930 bp plasmid that includes the human $VEGF_{165}$ coding gene, transcriptionally regulated by the cytomegalovirus promoter/enhancer, and a SV40 poly-A terminator. The placebo is obtained by excision of the human $VEGF_{165}$ coding gene. Preparation procedures, purification and quality control analyses were performed under GMP conditions (Bio Sidus, Buenos Aires, Argentina).

2.2. Surgical preparation and experimental protocol

This investigation conformed 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. Sheep received humane care throughout the study.

Thirty six Corriedale castrated male sheep weighing 28.4 ± 3.2 kg were operated on. After premedication with intramuscular acepromazine maleate (0.2 mg/kg), anesthesia was induced with intravenous sodium thiopental (20 mg/kg) and maintained with 2% halothane in pure oxygen under mechanical ventilation (Neumovent 910, Córdoba, Argentina). The electrocardiogram, heart rate and oxygen saturation (Novametrix 515A pulse oxymeter, Wallingford, CT) were monitored during surgery and recovery. After a sterile minithoracotomy at the 4th intercostal space, the left anterior descending artery (LAD) was dissected just proximal to the origin of the second diagonal branch and occluded for 90 min using a bulldog clamp. Anti-arrhythmic treatment with 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) was administered. At the onset of reperfusion, the 32 surviving sheep were randomized into one of 4 treatment groups: 1) intravenous recombinant human EPO (Bio Sidus, Buenos Aires, Argentina) 1000 units/kg in 50 ml vehicle solution (consisting of human albumin 20% 2.5 mg/ml, sodium chloride 3.2 mg/ml, D-mannitol 25 mg/ml, sodium monohydrogen phosphate dodecahydrate 4 mg/ml and anhydrous monobasic sodium phosphate 1.4 mg/ml) over 10 min plus $pVEGF_{165}$ 3.8 mg aliquotized in 10 intramyocardial injections along the infarct border (EPO + VEGF group, n = 8); 2) EPO plus empty plasmid 3.8 mg in 10 intramyocardial injections (EPO group, n = 8), 3) pVEGF plus vehicle solution (VEGF group, n = 8), and 4) empty plasmid plus vehicle solution (placebo group, n = 8). The nature of the injectates was blinded to all investigators until the end of data processing. The thoracotomy was then closed and cephalotin (30 mg/kg i.v.) was injected.

M-mode and bidimensional echocardiography was carried out at baseline and on days 3 and 15 postoperatively. Single photon emission computed tomography (SPECT) perfusion scans were obtained on days 3 and 15. On day 15, LV catheterization was performed and the animal was killed with an overdose of sodium thiopental followed by a bolus injection of potassium chloride to arrest the heart in diastole.

To assess apoptosis, 16 additional sheep were operated on and randomized to receive, upon reperfusion, EPO (n = 8) or vehicle (n = 8) as described above. These animals were killed 48 h (1 per group), 24 h (1 per group) and 8 h (6 per group) after treatment. The reasons for selecting these time points are explained later (see DNA fragmentation and activated caspase 3 in the Results section).

2.3. Infarct size

The heart was excised and the atria and right ventricle were removed. The LV was weighed, cut open parallel to the posterior interventricular sulcus and extended flat with the endocardial aspect exposed. Then, it was cut perpendicular to its apex–base axis at the level of the coronary occlusion. Two pieces thus resulted: one piece free of infarct and one piece containing the infarct. The latter was further cut parallel to the base every 5 mm. Finally, each of these pieces was divided into 3 blocks that were embedded in paraffin. In total, 24 to 32 large blocks per heart were thus obtained, ensuring the examination of the whole area of LV transverse sections. From each block, serial 5 μm thickness slices extending from the epicardium to the endocardium were cut and stained with hematoxilin and Masson's trichrome. The proportion of blue stained collagen with respect to the whole area of the LV, including that of the piece free of infarct, was determined by digital analysis (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD) in each tissue section at 4× magnification in high resolution scanned digital images and expressed as percent of total LV area. Non-blue stained areas within the ischemic-reperfused zone were considered as healthy myocardium, and therefore not counted as infarcted tissue.

2.4. Immunohistochemistry

Tissue sections from the infarct border and the adjacent viable myocardium were deparaffinized and brought to PBS, pH 7.2. After endogenous peroxidase blockade with 3% H_2O_2 in methanol and antigen retrieval pretreatment with citrate buffer in a microwave oven, the slides were incubated for 1 h with monoclonal antibodies against smooth muscle actin (BioGenex, San Ramon, CA) and Von Willebrand (Dako, Carpinteria CA) factors. They were then treated with biotinilated anti-mouse and anti-rabbit immunoglobulin antisera (Multilink, BioGenex) followed by peroxidase-labeled avidin, and revealed with AEC as chromogen.

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

In the 16 additional sheep, cells undergoing DNA fragmentation were detected in folmaldehyde-fixed samples of reperfused infarcts using the TUNEL assay (ApopTag Plus, Chemicon International, Temecula, CA). The samples were harvested from the infarct border adjacent to the viable myocardium. Tissue slices were processed following the manufacturer's instructions, revealed with peroxidase substrate over 3 min and counterstained with methyl green 0.5% p/v (BioGenex) for 10 min at room temperature. Positive nuclei were counted in a 0.8 mm^2 area at 40× magnification.

In these same sheep, caspase activity was assessed as described in the preceding paragraph employing a rabbit monoclonal antibody against activated caspase-3 (Cell Signaling Technology, Beverly, MA).

2.6. DNA fragmentation assay

In snap frozen myocardial samples of EPO-treated (n = 2) and placebo-treated (n = 2) sheep killed 8 h after AMI, DNA fragments were isolated using the technique described by Herrmann et al. [14]. Briefly, samples were pulverized and treated with lysis buffer (1% Nonidet P-40 in 20 mM EDTA and 50 mM Tris/HCl, pH 7.5). After centrifugation at 1600 *g* for 5 min, the supernatant was collected and the extraction was repeated with the same amount of lysis buffer. The supernatants were brought to 1% SDS and treated for 2 h with RNase A (final concentration: 5 μg/μl) at 56 °C followed by digestion with proteinase K (final concentration: 2.5 μg/μl) overnight at 37 °C. The genomic DNA was precipitated with 10 M ammonium acetate 0.5 vol and ethanol 2.5 vol, and the pellet dissolved in 10 μl water. Equal amounts of DNA for all the tested conditions were then analyzed by agarose gel electrophoresis (1.7%), followed by staining with ethidium bromide. Apoptotic cells from the collector duct of mammalian kidney were used as positive control. Normoperfused myocardium remote from the reperfused infarct was used as negative control.

2.7. Regional left ventricular function

M-mode, bidimensional and Doppler echocardiography (Sonos 5500, Hewlett Packard, Boston, MA) was performed under light sedation (diazepam, 10 mg intramuscular), with the conscious animal lying on its right lateral decubitus. Following standard guidelines, percent LV wall thickening (%WTh) and percent fractional shortening (%FS) were measured at the infarct border zone. LV diameters at end diastole (EDD) and end systole (ESD) were assessed using a single-plane multiple overlapped disks method and percent ejection fraction (%EF) was obtained. Cardiac output (CO) was calculated according to the following equation: CO (l/min) $= d^2 \times \pi/4 \times VTI \times HR$, where d is the LV outflow tract diameter in a pulsed Doppler long-axis parasternal view, VTI is the velocity–time integral obtained from a pulsed Doppler 5-chamber view and HR is heart rate [15]. Cardiac index (CI) in l/min/m^2 was obtained by dividing CO by body surface area (BSA). BSA was calculated as: BSA (m^2) = 0.084 × kg body weight$^{2/3}$.

2.8. Global left ventricular function

Immediately before euthanasia, LV catheterization was carried out under sedation (sodium thiopental 10 mg/kg), using a pressure tip catheter (Millar MikroTip, Millar Instruments, Houston, TX). The transducer's control unit (TC-510, Millar) was connected to a monitor and a computer with the aid of an A/D converter. After calibration, a cut down was performed to introduce the catheter in the left carotid artery and advance it to the aorta and the LV in order to record the aortic and LV pressure signals at 250 Hz frequency. LV peak systolic pressure (PSP), LV end diastolic pressure (EDP), aortic diastolic pressure (AoDP) and the maximum velocities of LV pressure rise and fall (dP/dtmax and dP/dtmin, respectively) were calculated using software developed in our laboratory. For each parameter, the values of all beats recorded during 10 s were averaged.

2.9. Myocardial perfusion

LV perfusion at rest was assessed with SPECT in an ADAC Vertex Dual Detector Camera System (Milpitas, CA). 99mTc-sestamibi was injected in the conscious state and acquisition was performed 2 h later under sedation with intravenous sodium thiopental (10 mg/kg at the beginning of the study and additional 1 mg/kg injections as required). Using the 20 segment LV model [16], the perfusion defect of each segment was assessed by 2 independent observers using a semiquantitative score in which 0 corresponds to normal perfusion, 4 to perfusion absence and 1, 2 and 3 to mild, moderate and severe perfusion defect, respectively. Finally, the summed rest score (SRS), resulting from the sum of the 20 individual scores, was calculated and considered to represent the overall LV perfusion defect.

2.10. VEGF gene expression

Human $VEGF_{165}$ DNA, mRNA and protein were assessed at 15 days post-treatment in transfected myocardium samples frozen in liquid nitrogen and stored at −80 °C.

In 2 animals per group, polymerase chain reaction (PCR), reverse transcriptase (RT)-PCR and Western Blot analysis were performed. The samples were pulverized in liquid nitrogen to obtain DNA, RNA and protein.

PCR: total DNA was isolated (Qiagen, Hilden, Germany) and quantitated (A260 nm spectrophotometry). Amplification was done using GeneAmp PCR core kit (Perkin-Elmer, Boston, MA) and previously reported specific primers [17].
RT-PCR: total RNA was extracted (Trizol, Gibco BRL, Grand Island, NY), treated with DNAse I (Promega, Madison, WI), quantitated, reverse-transcripted (random hexamers, Perkin-Elmer, Boston, MA) and amplified by PCR. In each reaction, GAPDH gene amplification was performed as endogenous control. Amplification products were run in 1.5% agarose gel with ethidium bromide and visualized.
Western blot: total proteins were extracted and quantified using a Bradford assay (Bio-Rad, Hercules, CA). Sample proteins were resolved under non-reducing conditions on a 12% SDS-polyacrylamide gel. Immunoblotting was performed using a monoclonal antibody against human $VEGF_{165}$ (MAB 293, R&D Systems, Minneapolis, MN). The membrane was then probed with a secondary anti mouse antibody conjugated to horseradish peroxidase P260 (Dako), developed with chemiluminescence (ECL, RPN 2106, Amersham, IL) and exposed to X ray (BioMax, Kodak, NY). A human $VEGF_{165}$ standard (293-VE, R&D Systems) was used as positive control. Immunoblotting for sarcomeric α-actin was performed as an internal loading control.

2.11. Statistical analysis

One-way ANOVA–Newman–Keuls test was used to analyze infarct size, capillary and arteriolar densities and global LV function parameters. Regional LV function parameters, perfusion defect and hematologic variables were analyzed using two-way ANOVA followed by post-hoc analysis with Bonferroni test. Comparison of apoptotic nuclei between animals receiving EPO and animals not receiving EPO was made using a Student's *t*-test. Values were expressed as mean ± SD. Statistical significance was established for $p<0.05$.

3. Results

3.1. Mortality

Of 52 operated sheep, 4 (7.7%) died at onset of reperfusion due to irreversible ventricular fibrillation. None of them had received treatment prior to death.

3.2. Infarct size

LV weight was similar in all groups (VEGF + EPO: 87.4 ± 10.1 g; EPO: 83.3 ± 9.9 g; VEGF: 86.8 ± 8.8 g; placebo: 87.2 ± 8.4 g, p = NS). Infarct size, as percent fibrosis of total left ventricular mass, was smaller in the 3 treated groups (VEGF + EPO: 8 ± 1%; EPO: 13 ± 4%; VEGF: 16 ± 5%) compared to placebo (25 ± 7%, $p<0.001$). However, the infarct size observed in the combined EPO + VEGF treatment group was significantly smaller ($p<0.05$) than those of the EPO and the VEGF groups (Fig. 1).

3.3. Regional left ventricular function

Fig. 2a shows %WTh results. Three days after treatment, both groups receiving EPO displayed higher %WTh (EPO + VEGF: 58 ± 26%; EPO: 59 ± 26%) than the other two groups (placebo: 32 ± 17% and VEGF: 27 ± 13%, $p<0.05$). On day 15, these differences disappeared because %WTh tended to decrease in the EPO-treated groups (EPO + VEGF: 41 ± 22%; EPO: 45 ± 23%) while it increased in the placebo- (36 ± 10%) and VEGF-treated (51 ± 22%) groups. In the case of %FS (Fig. 2b), both groups receiving pVEGF had significantly higher values ($p<0.05$) than placebo at the end of the study (EPO + VEGF: 42.6 ± 5%; VEGF: 43.4 ± 5%; EPO: 36 ± 7%; placebo: 33 ± 6%). The combined treatment was also protective at the earlier time point (day 3 post-AMI), %FS being 36 ± 9% in the VEGF + EPO group and 27 ± 6% in the placebo group ($p<0.05$). Paired analysis revealed that EPO prevented %WTh and %FS from decreasing early after RAMI. On the other hand, in the groups receiving pVEGF, protection was attained only at the later time point (day 15), after gene expression had taken place.

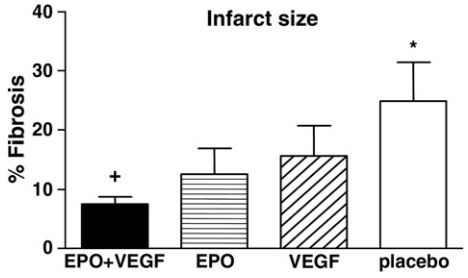

Fig. 1. Infarct size. Percent fibrosis of left ventricular mass was smaller with all three treatments, but the association of plasmid-mediated vascular endothelial growth factor gene transfer and erythropoietin (VEGF + EPO) limited infarct size to a significantly larger extent than individual VEGF or EPO. *$p<0.001$ vs. all groups. $^{+}p<0.05$ vs. EPO and VEGF.

3.4. Global left ventricular function

Table 1 lists the values for LV diameters, volumes and indexes of global LV performance on day 15 post-treatment. Except for EDV and ESV of sheep treated with EPO only, none of them (i.e. EDD, ESD, %EF, PSP, EDP, AoDP, dP/dtmax and dP/dtmin) showed significant differences between groups.

3.5. Myocardial perfusion

The overall LV perfusion defect was not different between groups either on day 3 or at the end of the study, as indicated by unpaired analysis of SRS values. However, paired analysis revealed that it increased between day 3 and day 15 post-treatment in the placebo group (from 19 ± 7 to 22 ± 7, $p<0.05$) but not in the other 3 groups

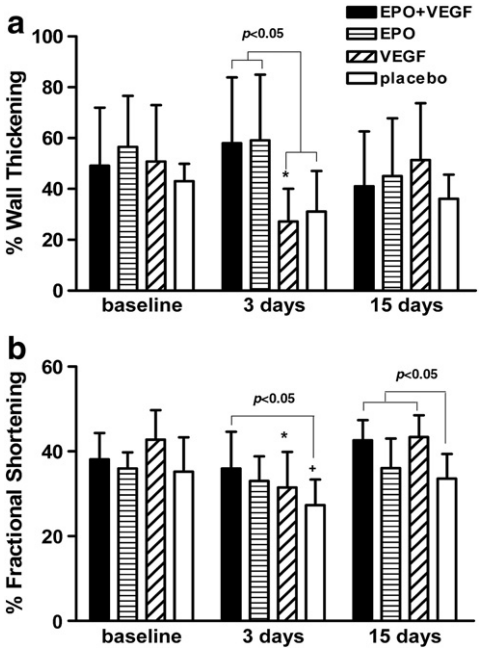

Fig. 2. Regional left ventricular function. Percent wall thickening at the infarct border (a) on day 3 after reperfused infarction was preserved only in the 2 groups receiving erythropoietin (EPO) treatment. At the end of the study (day 15) no significant differences were observed. *$p<0.05$ vs. VEGF at baseline and VEGF on day 15. Percent fractional shortening (b) was significantly higher with respect to placebo on days 3 and 15 after reperfused infarction with the combined VEGF + EPO treatment, and only on day 15 with VEGF gene transfer alone. *$p<0.05$ vs. VEGF at baseline and VEGF on day 15. $^{+}p<0.06$ vs. placebo at baseline.

Table 1
Global left ventricular function parameters on day 15 after treatment.

Parameter	EPO + VEGF	EPO	VEGF	Placebo
EDD (mm)	39.8 ± 4.4	39.9 ± 3	38 ± 4	35.4 ± 2.4
ESD (mm)	22.9 ± 3.2	25.5 ± 3.5	21.4 ± 1.8	23.5 ± 3.1
EDV (ml)	57.5 ± 13.5	62 ± 11.8*	57.3 ± 7.6	45.5 ± 12.3
ESV (ml)	27.8 ± 7.5	33.1 ± 8.6*	29.3 ± 3.5	23.8 ± 7.9
EF%	52 ± 7	49 ± 11	48 ± 8	46 ± 10
CI (l/min/m^2)	3.2 ± 1	3.6 ± 1	2.9 ± 1	3.5 ± 1
PSP (mm Hg)	124 ± 17	115 ± 27	124 ± 24	129 ± 18
AoDP (mm Hg)	94 ± 16	90 ± 22	102 ± 19	98 ± 13
EDP (mm Hg)	10 ± 3	13 ± 8	13 ± 4	11 ± 3
dP/dtmax (mm Hg/s)	2084 ± 538	2219 ± 972	2270 ± 759	2901 ± 1490
dP/dtmin (mm Hg/s)	−2737 ± 895	−2752 ± 710	−3565 ± 1535	−3127 ± 681

AoDP: aortic diastolic pressure; CI: cardiac index; dP/dtmax: maximum rate of left ventricular pressure raise; dP/dtmin: maximum rate of left ventricular pressure decay; EDD: end diastolic diameter; ESD: end systolic diameter; EDP: end diastolic pressure; EDV: end diastolic volume; ESV: end systolic volume; EF: ejection fraction; EPO: erythropoietin; PSP: peak systolic pressure; VEGF: vascular endothelial growth factor.
* $p<0.05$ vs. placebo.

(VEGF: from 21 ± 6 to 21 ± 8; EPO: from 21 ± 7 to 21 ± 9; VEGF + EPO: from 20 ± 9 to 21 ± 10), indicating that EPO and VEGF (alone or combined), but not placebo, were able to preserve the level of myocardial perfusion observed early after infarction (Fig. 3).

3.6. Capillaries and arterioles

Reperfused myocardial infarction exhibits a marked histologic heterogeneity with areas at diverse stages of evolution, ranging from normality to consolidated fibrosis, coexisting within the same topographic zone. This characteristic of reperfused AMI is an important limitation that prevents the accurate assessment of microvessel density. Fig. 4 illustrates a reperfused infarct reconstructed from slices spanning the whole wall thickness, showing the heterogeneous distribution of microvessels within the same infarct.

3.7. DNA fragmentation and activated caspase 3

To evaluate the effect of EPO on apoptosis, 4 additional sheep were operated on and randomized into a placebo group (n = 2) and an EPO-treated group (n = 2). We killed one animal per group 48 and 24 h post-reperfusion, but failed to find noticeable TUNEL-positivity at either time points. Assuming that apoptosis occurred at an earlier time, we decided to operate another 12 sheep (6 EPO-treated and 6 placebo-treated animals) and killed them 8 h after reperfusion. In these sheep, a blinded analysis showed that the number of TUNEL-positive cells was significantly lower in the EPO-treated sheep (12.8 ± 19.6 per mm^2) than in the placebo group (86.5 ± 31.8, $p<0.004$). The DNA fragmentation assays carried out in 2 animals per group showed the typical "laddering" pattern (Fig. 5). Only occasionally were positive reactions for activated caspase-3 observed in both groups.

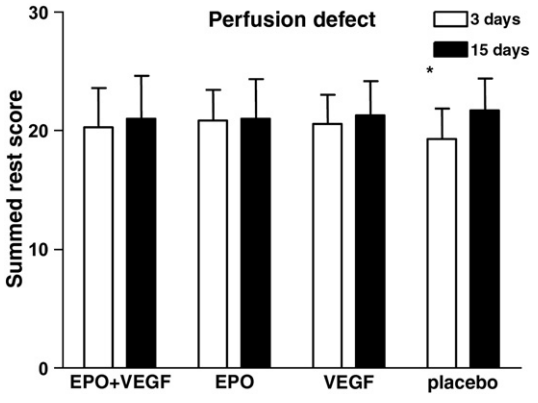

Fig. 3. Myocardial perfusion. The perfusion defect of the left ventricle increased between day 3 and day 15 post-infarction in the placebo group, but not in the other groups, indicating that erythropoietin (EPO), vascular endothelial growth factor (VEGF) gene transfer or the association of both agents (EPO + VEGF) equally preserved myocardial perfusion. *$p<0.05$ vs. 15 days.

3.8. Transgene expression

On day 15 post-transfection, Western blot analysis for human $VEGF_{165}$ protein was positive in the myocardium of VEGF-treated animals. A faint positive reaction was observed in samples from animals receiving placebo, probably due to cross-reaction with ovine VEGF. Reverse transcription PCR (RT-PCR) with specific primers for human VEGF mRNA was negative on day 15 post-transfection, confirming the transient nature of VEGF expression described in a previous study [4] (Fig. 6).

3.9. Hematologic parameters

To evaluate whether recombinant human EPO was biologically active in sheep, we determined the hematocrit and platelet count at different time points in EPO-treated sheep (n = 16). Hematocrit increased significantly at 7 (36% ± 2), 10 (36% ± 3) and 14 (37% ± 3) days with respect to baseline (34% ± 3, $p<0.01$). Platelet count increased on day 10 after treatment from $4.6\times10^5 \pm 1.5\times10^5$ per mm^3 to $5.9\times10^5 \pm 1.7\times10^5$ per mm^3 ($p<0.05$), and returned to baseline on day 15.

4. Discussion

4.1. Infarct size

Based on previous studies in pigs and sheep showing that direct plasmid-mediated VEGF gene transfer attenuates ischemia and reduces infarct size by combining angio-arteriogenic, antifibrotic and cardiomyogenic effects [4,17–19] and that EPO exerts anti-apoptotic actions in animal models of AMI [7–11], we aimed to assess if the association of both agents would limit reperfused infarct size to a larger extent than any of them individually. Our results showed that all 3 treatments reduced the area of fibrosis, but that, as hypothesized, this was significantly smaller with combined VEGF and EPO than with either intervention alone. The infarct limiting effect achieved with VEGF + EPO was approximately 38% and 50% more efficient than that induced by pVEGF and EPO, respectively. Given the direct relationship between infarct size and severity of post-infarct ventricular remodeling, this result may be of potential clinical relevance.

4.2. Dosage

We used 3.8 mg of pVEGF because this dose proved to be effective in our previous studies on porcine and ovine models of coronary artery disease [4,17–19].

With regard to EPO, although massive doses (3000 to 5000 IU/kg/-day over several days) have been recommended, we decided to use 1000 IU/kg in a single administration because in a previous study using 3000 IU/kg on 3 consecutive days in sheep with reperfused AMI, we observed no infarct size reduction and even a detrimental effect on LV function [20]. Our results indicate that the dosage selected for the present study induced a noticeable anti-DNA fragmentation effect on ovine reperfused AMI. Moreover, recent clinical studies have shown that lower EPO doses have no effect on infarct size

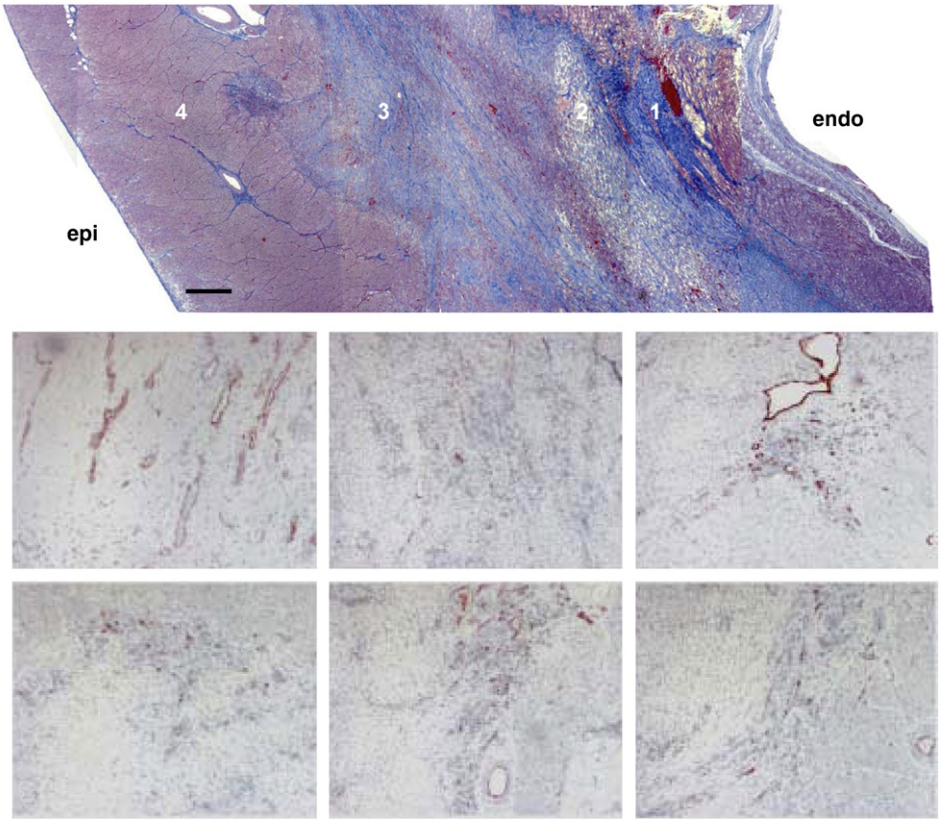

Fig. 4. Histologic heterogeneity of reperfused myocardial infarction. (a) Reconstruction of a 15-day old reperfused infarct from slices spanning the whole wall thickness from epicardium (epi) to endocardium (endo). Zones of intense fibrosis (1) coexist with areas of inflammation and angiogenesis (2), mild fibrosis (3) and normal myocardium (4). Masson's trichrome staining. Bar: 500 μm. (b) Diverse slices from the same infarct show great differences in microvascular density. Hematoxilin plus anti-Von Willebrand factor immunostain. Bar: 50 μm.

limitation [21–23]. The dose used was biologically active, on account that the hematocrit and platelet count increased slightly, though significantly. However, no undesired side effects would be expected from these changes, given that the hematocrit did not increase beyond normal ovine physiological levels and that the increase in platelet count was only transient.

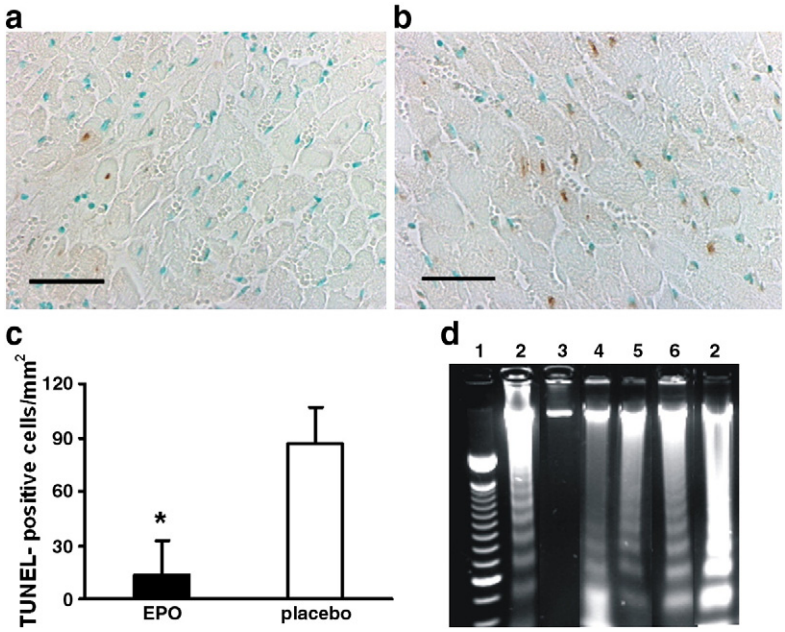

Fig. 5. DNA fragmentation. In samples of reperfused myocardial infarcts from an erythropoietin (EPO)-treated (a) and a placebo-treated (b) sheep it can be seen that TUNEL-positive reactions predominate in the latter. Bars: 20 μm. (c) TUNEL-positive cells were significantly decreased in EPO-treated sheep. (d) DNA fragmentation assays. Lane 1: molecular mass DNA marker; lane 2: positive control; lane 3: negative control; lanes 4 and 5: reperfused infarct from 2 EPO-treated sheep; lanes 6 and 7: reperfused infarct from 2 placebo-treated sheep.

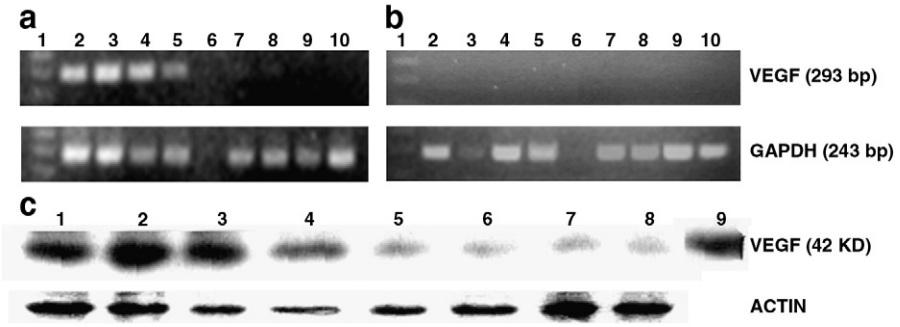

Fig. 6. Vascular endothelial growth factor (VEGF) gene expression in myocardium transfected with the human $VEGF_{165}$ gene. (a) Polymerase chain reaction (PCR) for $VEGF_{165}$ DNA (upper panel) and GAPDH DNA (lower panel). Lane 1: molecular weight marker; lanes 2 and 3: VEGF-treated sheep; lanes 4 and 5: VEGF + erythropoietin (EPO)-treated sheep: lane 6: negative control; lanes 7 and 8: EPO-treated sheep; lanes 9 and 10: placebo sheep. (b) Reverse-transcription (RT)-PCR for $VEGF_{165}$ mRNA (upper panel) and GAPDH mRNA (lower panel). Lanes as in a. (c) Western blot for the human $VEGF_{165}$ protein (upper panel) and sarcomeric α-actin (lower panel). Lanes 1 and 2: VEGF-treated sheep; lanes 3 and 4: VEGF + EPO-treated sheep; lanes 5 and 6: EPO-treated sheep; lanes 7 and 8: placebo sheep; lane 9: positive control (human $VEGF_{165}$).

4.3. DNA fragmentation, a hallmark of late apoptosis

Apoptosis or, at least, activation of the apoptotic pathway, has been shown to be a relevant cell death mechanism in ischemia-reperfusion [24–26]. Some authors claim that apoptosis occurs only after reperfusion [27,28] while others indicate that reperfusion just accelerates apoptosis in cells committed during ischemia to undergo apoptotic death [25]. Since apoptosis is an active process, its development needs oxygen, ATP and cytokines [29]. Thus, although ischemia may trigger the apoptotic pathway, reperfusion completes DNA fragmentation and the morphological changes observed in the final stages of apoptosis [30].

In necropsies from patients dying after reperfused AMI, Saraste et al. observed apoptotic cardiomyocytes in the peri-infarct zone. These cells represented up to 5% of all the cardiomyocytes in each microscopic field [31]. In other studies on necropsies of patients who died after AMI, myocyte apoptosis ranged from 8% [32] to 12% [33]. Our results are consistent with these figures since: 1) infarct size reduction in the EPO + VEGF group was 8% greater than in sheep receiving VEGF alone; and 2) in sheep treated only with EPO, infarct size was roughly 12% smaller than in those receiving placebo. On the basis of these percentages, it may be speculated that EPO reduced infarct size by preventing apoptosis. In addition, the time course of apoptotic death in our sheep is consistent with previous observations showing that some molecular events, namely caspase-3 activation and cleavage of poly-(ADP-ribose) polymerase, occur early during ischemia, and some, like DNA fragmentation, during late reperfusion [33]. Accordingly, 8 h after AMI (6 h and 30 min after reperfusion) we observed profuse DNA fragmentation but almost no caspase-3 activity. Moreover, we detected DNA fragmentation 8 h after AMI but not after 24 or 48 h, in agreement with Veinot et al., who reported that apoptosis is not only the early and predominant form of cell death in infarcted human myocardium, but its development is accelerated by reperfusion [34]. Nevertheless, since a cause–effect relationship between reduced DNA fragmentation and limited infarct size cannot be directly inferred from our data, these interpretations should be considered with caution.

4.4. Myocardial perfusion

A mechanism of cardioprotection shared by VEGF and EPO is neovascular growth [12,35]. Given the marked histologic heterogeneity of reperfused infarcts, it is extremely difficult to reliably assess microvascular density. As stated above and illustrated in Fig. 4, zones with profuse angiogenesis coexisted anarchically with fibrotic areas almost devoid of microvessels and areas of inflammation. As a consequence, microvessel density varied substantially depending on the selected zone, producing erratic results.

We do have, however, a reliable estimation of the overall perfusion defect. Paired analysis of the SPECT scans revealed that myocardial perfusion worsened only in placebo sheep, remaining unchanged in the rest of the groups. On this ground, it may be speculated that rather than inducing angiogenesis, the 3 treatments could have prevented microvascular loss, probably by attenuating apoptosis of endothelial and smooth muscle cells.

4.5. LV function

Results showed that EPO exerted an early protective effect on LV function, since 3 days after AMI, only the groups receiving EPO preserved %WTh and %FS at the peri-infarct zone. Conversely, the effect of VEGF was first evident 15 days post-AMI, due to the time needed for gene expression. It should be noted that, at that time point, the human VEGF protein, but not VEGF mRNA, was detected, in agreement with previous data on $pVEGF_{165}$ expression in ovine myocardium [4]. On day 15 post-AMI, either VEGF alone or the combined treatment were similarly beneficial in terms of regional %FS.

The substantial reduction in infarct size accompanied by moderate improvements in regional LV function and essentially no changes in global LV performance might be attributed to persistent hibernation in zones of salvaged myocardium. In agreement with this assumption, in sheep with permanent LAD occlusion, we have observed that VEGF gene transfer limited infarct size but did not improve LV function 15 days after AMI [4]. However, in a later study on the same ovine model and with the same treatment, but with 60 days follow-up, %WTh and %FS were significantly higher in VEGF-treated than in placebo-treated animals, suggesting functional recruitment of areas that were earlier hibernated (unpublished results). With regard to the increased EDV and ESV observed in the EPO-treated group, it is unlikely that they represent genuine remodeling, given that the time elapsed between the infarct and the end of the follow-up period was not long enough to allow the histological changes characterizing remodeling. Effectively, ovine infarcts involving approximately 25% of the LV mass (as those of the present placebo-treated sheep) develop significant remodeling in about 10 weeks [36].

4.6. Clinical aspects

On account of the growing availability and use of early percutaneous revascularization procedures, the prevalence of reperfused myocardial infarctions in the daily clinical practice is increasing. Our results suggest that, in this setting, combined intramyocardial pVEGF transfer and intravenous EPO might help to enhance myocardial salvage.

It should be noticed that cardioprotective effects of EPO as well as of VEGF gene transfer independently have been reported. The

advantage of the combined therapy is that it affords a steady protection, starting at an early stage owing to EPO and persisting at later time points due to VEGF effects. The time course of regional LV performance supports this reasoning, on account that it improved as early as 3 days post-RAMI only in the groups receiving EPO and stayed improved 15 days in the VEGF and EPO + VEGF groups.

A surgical approach was used to inject the plasmid. However, at present, percutaneous injection catheters designed to deliver transendocardial gene therapy are being used in clinical trials of chronic myocardial ischemia [37–40], making the proposed treatment technically feasible. Nevertheless, safety issues associated with puncturing the myocardium within the early phase of reperfusion should be appropriately assessed before testing this treatment in humans.

5. Conclusion

In adult sheep with reperfused acute myocardial infarction, the association of intramyocardial plasmid-mediated human $VEGF_{165}$ gene transfer plus intravenous erythropoietin 1000 IU/kg immediately after reperfusion decreased infarct size to a greater extent that either individual treatment 15 days after infarction and improved regional, though not global, left ventricular function. These cardioprotective effects may be clinically interesting to enhance the benefits of early revascularization in patients with acute myocardial infarction.

Disclosures

One of the authors (AB) is a senior scientist at Bio Sidus (Buenos Aires, Argentina), the biotechnology company that developed the plasmid-VEGF used in the study. The rest of the authors have nothing to disclose.

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