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ORIGINAL ARTICLE

Mesenchymal stromal cells overexpressing vascular endothelial growth factor in ovine myocardial infarction

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Mesenchymal stromal cells (MSCs) are cardioprotective in acute myocardial infarction (AMI). Besides, we have shown that intramyocardial injection of plasmid-$VEGF_{165}$ (pVEGF) in ovine AMI reduces infarct size and improves left ventricular (LV) function. We thus hypothesized that MSCs overexpressing $VEGF_{165}$ (MSCs-pVEGF) would afford greater cardioprotection than non-modified MSCs or pVEGF alone. Sheep underwent an anteroapical AMI and, 1 week later, received intramyocardial MSCs-pVEGF in the infarct border. One month post treatment, infarct size (magnetic resonance) decreased by 31% vs pre-treatment. Of note, myocardial salvage occurred predominantly at the subendocardium, the myocardial region displaying the largest contribution to systolic performance. Consistently, LV ejection fraction recovered to almost its baseline value because of marked decrease in end-systolic volume. None of these effects were observed in sheep receiving non-transfected MSCs or pVEGF. Although myocardial retention of MSCs decreased steeply over time, the treatment induced significant capillary and arteriolar proliferation, which reduced subendocardial fibrosis. We conclude that in ovine AMI, allogeneic VEGF-overexpressing MSCs induce subendocardial myocardium salvage through microvascular proliferation, reducing infarct size and improving LV function more than non-transfected MSCs or the naked plasmid. Importantly, the use of a plasmid rather than a virus allows for repeated treatments, likely needed in ischemic heart disease.

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INTRODUCTION

Despite significant advances in early reperfusion strategies and medical treatment, ischemic heart disease remains the leading cause of death worldwide.[1] Its most severe complication is acute myocardial infarction (AMI), which leads to ventricular remodeling, a process characterized by progressive replacement of the remaining contractile cells by fibrosis.[2] The loss of myocytes and its substitution by noncontractile tissue leads to heart failure. The extent of remodeling and hence the chances of evolving to heart failure are largely dependent on infarct size.[3–5] Therefore, therapeutic strategies aimed at reducing ischemic damage by transferring angiogenic genes or implanting stem cells of diverse origin are being increasingly investigated.

Although promising results have been reported for either gene or cell therapy used as single therapies in laboratory rodents and large mammalian models of cardiac disease, most clinical trials show that the effect on left ventricular (LV) function is rather modest or even undetectable.[6] A plausible strategy to enhance the therapeutic efficacy is the combination of both approaches, through the genetic modification of stem cells to overexpress angiogenic growth factors or signaling molecules that can enhance their regenerative potential.[7–13]

On account of their easy isolation and *ex vivo* expansion, their amenability for genetic modification and their potential for allogeneic utilization, bone marrow mesenchymal stromal cells (MSCs) constitute one of the cell types most consistently used in pre-clinical and clinical studies of cardiac regeneration.[14,15]

On the other hand, we have shown in pigs and sheep with AMI that the intramyocardial injection of a plasmid encoding human $VEGF_{165}$ in the border zone reduces infarct size by promoting capillary and arteriolar growth (angio-arteriogenesis), limiting collagen deposition and inducing cardiomyogenesis.[16–19]

We thus hypothesized that the modification of MSCs to overexpress $VEGF_{165}$ would result in greater effects on infarct size and LV function, provided that the cells efficiently carry and express VEGF and that, additionally, they secrete other pro-angiogenic and mitogenic factors that acting in a paracrine manner would produce greater improvement in LV function in a large mammalian model of myocardial infarction.

RESULTS

VEGF-overexpressing MSCs reduce infarct size and induce tissue salvage predominantly in the subendocardial myocardium

One week after undergoing a LV anteroapical AMI, adult sheep ($n=8$) were injected in the infarct border with 2×10^7 MSCs transfected with a plasmid encoding human $VEGF_{165}$. (MSCs-pVEGF group). Equal number of sheep received either non-transfected cells (MSCs group) or 3.8 mg of the naked plasmid (pVEGF group) or phosphate-buffered saline (placebo group). To compare pre- vs post-treatment infarct size, magnetic resonance imaging (MRI) was performed at 3 and 30 days after AMI.

Infarct size was reduced by 31% in the MSCs-pVEGF group (from 13 ± 5 to $9\pm4\%$, $P<0.01$) and by 21% in pVEGF (from 14 ± 6 to $11\pm3\%$, $P<0.05$). In the MSCs and placebo groups, infarct size showed a non-significant tendency to decrease (MSCs: from 11 ± 3 to $8\pm3\%$, $P=$NS; placebo: 13 ± 4 to $12\pm5\%$, $P=$NS) (Figure 1a). Pre- and post-treatment MRI images are shown in Figures 1b–e.

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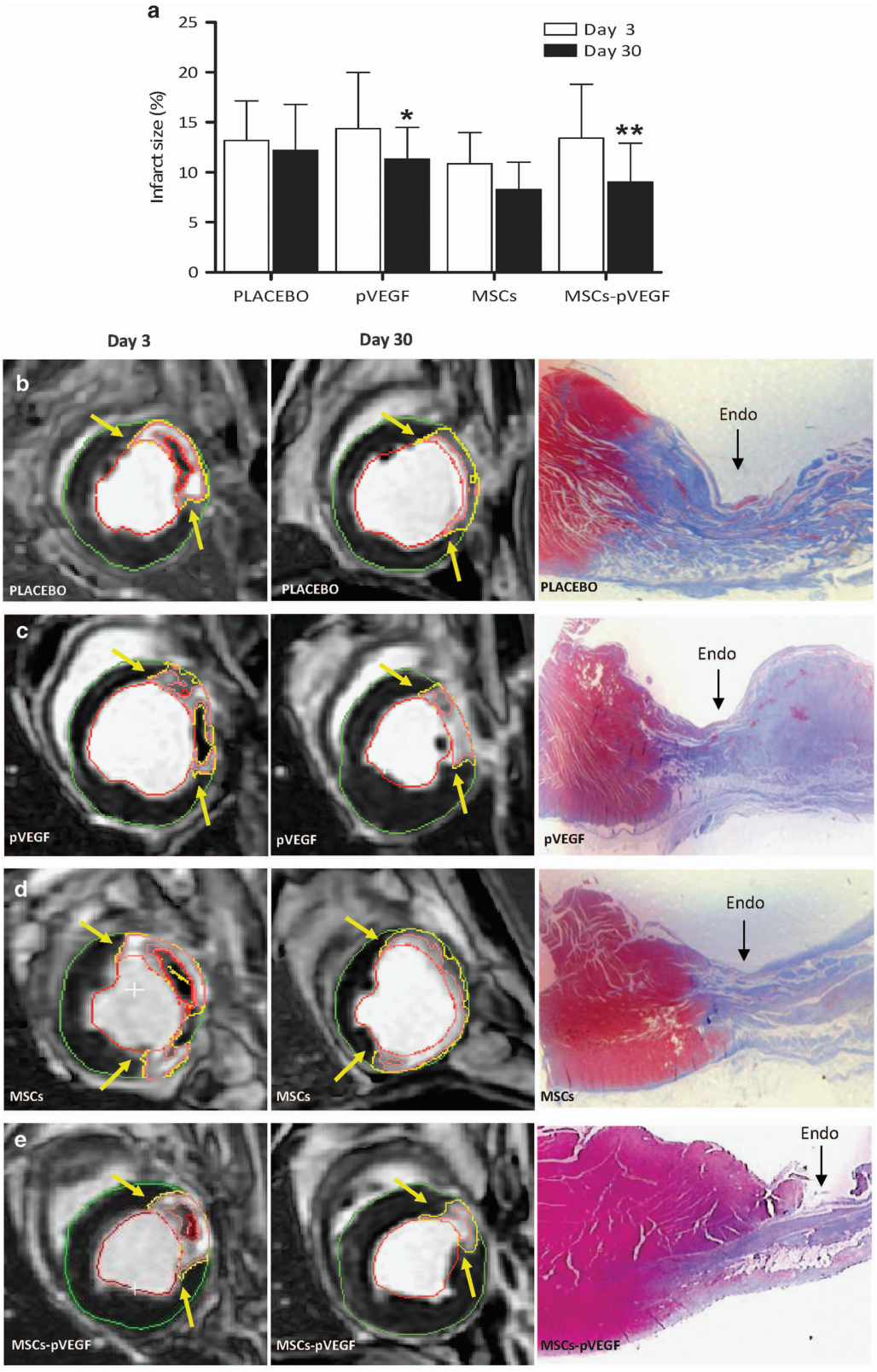

Figure 1. Infarct size. (**a**) Between day 3 post infarction and day 30 post treatment, infarct size as percent total LV mass displayed a significant 31% decrease in the group treated with VEGF-transfected MSCs (MSCs-pVEGF) and 21% decrease in the group treated with the naked plasmid (pVEGF). (**b–e**) MR images of a short axis slice at day 3 post infarction (left) and at day 30 post treatment (mid) in a sheep treated with placebo (**b**), pVEGF (**c**), MSCs (**d**) and MSCs-pVEGF (**e**). The arrows mark the infarct limits. The right panels in **b**, **c**, **d** and **e** show the corresponding infarct borders in slices stained with Masson's trichrome (scar in blue and viable myocardium in red). Consistent with the MR images, the slices show that in MSCs-pVEGF, but not in placebo, MSCs and pVEGF, myocardial salvage occurs predominantly at the subendocardial level (Endo). *$P < 0.05$ vs day 3; **$P < 0.01$ vs day 3. Mean ± s.d.

In agreement with the MR images, analysis of transmural sections stained with Masson's trichrome consistently showed that in MSCs-pVEGF contractile tissue salvage occurred predominantly at the subendocardial myocardium. This feature was not observed in the remaining groups (Figures 1b and e). Consistent with this observation, the fibrotic area in the peri-infarct as percent of an area comprised at both sides of the infarct border was significantly less in the MSCs-pVEGF group (35.5 ± 15.6%) than in the other groups (MSCs: 80.4 ± 21%; pVEGF: 72.6 ± 24.2%; placebo: 75.7 ± 24%, all $P < 0.01$ vs MSCs-pVEGF).

VEGF-overexpressing MSCs improve LV function by reducing end-systolic volume (ESV)

Bidimensional echocardiography showed that LV end-diastolic volume (EDV) increased only in the placebo group from 69.4 ± 6.2 ml at 3 days post AMI to 84.2 ± 5 ml at 30 days post treatment ($P < 0.05$). There were no significant EDV changes in the other three groups, this indicating that all treatments except placebo prevented LV dilation (Figure 2a). ESV increased significantly early after AMI in all experimental groups (placebo from 22.4 ± 7.0 to 43 ± 13.9 ml, $P < 0.01$; pVEGF from 20.2 ± 3.8 to 33.5 ± 7 ml, $P < 0.01$; MSCs from 23.6 ± 9.2 to 34.9 ± 8.4 ml, $P < 0.05$; MSCs-pVEGF from 29.8 ± 7.9 to 41.7 ± 9.9 ml; $P < 0.05$). At 30 days post treatment, ESV decreased only in the MSCs-pVEGF group (from 41.7 ± 9.9 to 32.8 ± 8.0 ml, $P < 0.05$) (Figure 2b). LV percent ejection fraction (%EF) decreased significantly early after AMI in all experimental groups. At 30 days follow-up, %EF improved significantly only in the MSCs-pVEGF group (from 33.8 ± 10.4 to 53.4 ± 6.9% ($P < 0.05$). In the remaining three groups, %EF showed a tendency to increase with regard to the post-AMI values, (placebo from 38.4 ± 8.2 to 43.9 ± 3.1%; pVEGF from 42.8 ± 12.4 to 53.8%; MSCs from 41.6 ± 6.7 to 51.9%), but in no case did the differences achieve statistical significance (Figure 2c).

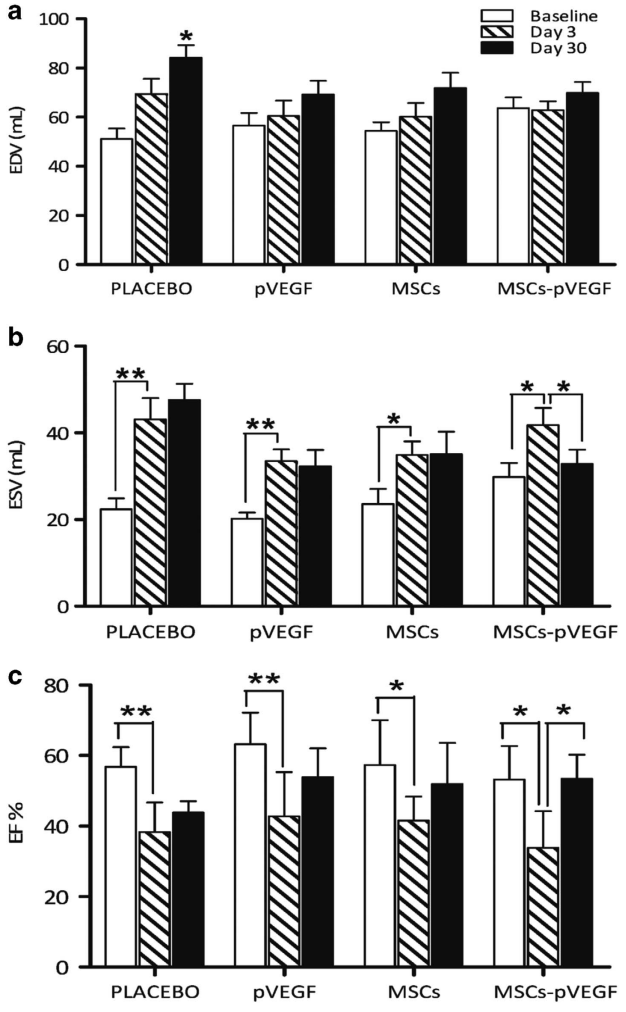

Figure 2. LV function at baseline, 3 days post infarction and 30 days post treatment. (**a**) EDV increased significantly over time only in the placebo group. (**b**) Post-infarction ESV increased significantly with regard to baseline in all groups, but decreased significantly after treatment (day 30) only in the group treated with VEGF-transfected MSCs (MSCs-pVEGF). (**c**) Post-infarction %EF decreased significantly with regard to baseline in all groups, and increased significantly after treatment (day 30) only in the MSCs-pVEGF group. *$P < 0.05$; **$P < 0.01$. Mean ± s.d.

Table 1 lists the values for peak systolic pressure, end-diastolic pressure, dP/dt_{max} and dP/dt_{min}. obtained at 30 days post treatment through LV catheterization. There were no significant differences among groups in any of the analyzed parameters.

VEGF-overexpressing MSCs induce both capillary and arteriolar proliferation

In sheep hearts studied at 30 days post treatment, there were no differences in arteriolar and capillary densities among groups (Table 2). In contrast, in additional sheep killed at 10 days post treatment, capillary density was significantly higher in MSCs-pVEGF (2576 ± 522 capillaries per mm^2) and pVEGF (2651 ± 319) groups as compared with placebo (1719 ± 158, $P < 0.01$ vs MSCs-pVEGF and pVEGF). On the other hand, arteriolar density at the same time point was higher in MSCs-pVEGF (24 ± 8 arterioles per mm^2) and MSCs groups (21 ± 4) than in placebo (7 ± 2, $P < 0.01$ vs MSCs-pVEGF and pVEGF). Thus, the only group that combined significantly higher arteriolar *and* capillary densities was the MSCs-pVEGF group (Figure 3).

To assess for cycling cardiomyocytes, we performed immunohistochemistry against the Ki67 antigen in these additional sheep. We found that Ki67-positive cardiomyocyte nuclei were exceptional, not exceeding 10 per million in any of the experimental groups.

Cell retention in the injected myocardium decreases steeply over time

For cell tracking, additional female sheep were injected with male VEGF-transfected MSCs previously marked with the fluorescent dye PKH26, and killed at three time points after injection. The SRY gene in the peri-infarct region was present at 7 days post treatment, decayed at 21 days and was hardly detectable at 30 days (Figure 4a). This was consistent with the histological analysis of peri-infarct cryosections, which showed that PKH26-marked VEGF-transfected MSCs were clearly present at 7 days but extremely scarce at 30 days post treatment (Figure 4b).

Myocardial expression of human VEGF in MSCs-pVEGF-treated sheep persists for at least 30 days after treatment

Human VEGF mRNA was assessed by RT-qPCR in myocardial samples from MSCs-pVEGF-treated sheep at 7, 10, 21 and 30 days post treatment. Transgene expression peaked at 10 days and decayed thereafter, still being present at end of follow-up (Figure 5).

DISCUSSION

Our results show that in sheep with AMI, the intramyocardial injection of allogeneic MSCs transfected with a plasmid encoding $VEGF_{165}$ reduces infarct size and improves LV function to a greater extent than non-transfected MSCs or the naked plasmid by promoting proliferation both of capillaries and arterioles.

Table 1. Left ventricuar hemodynamic parameters at 30 days post treatment

	Placebo	*pVEGF*	*MSCs*	*MSCs-pVEGF*
PSP (mm Hg)	87.1 ± 33.8	79 ± 23.6	96.7 ± 11.1	89.9 ± 4.2
EDP (mm Hg)	7.9 ± 1.8	5.3 ± 2.2	4.7 ± 1.2	4.6 ± 1.6
dP/dt_{max} (mm Hg s^{-1})	1033.7 ± 629.8	1103.6 ± 730.2	1150.1 ± 516.9	1129.2 ± 212.5
dP/dt_{min} (mm Hg s^{-1})	− 2264.8 ± 1423.2	− 1915.1 ± 1156	− 1992.6 ± 237.2	− 1795 ± 227.3

Abbreviations: dP/dt_{max}, maximum rate of left ventricular pressure increase; dP/dt_{min}, maximum rate of left ventricular pressure decay; EDP, left ventricular end-diastolic pressure; MSCs, mesenchymal stromal cells; PSP, left ventricular peak systolic pressure; pVEGF, plasmid encoding human vascular endothelial growth factor. No significant differences were found in any hemodynamic parameters at the end of the follow up. Mean ± s.d.

Table 2. Arteriolar and capillary density at 30 days post-treatment

	Placebo	*pVEGF*	*MSCs*	*MSCs-pVEGF*
Arterioles per mm^2	6 ± 3	5 ± 2	8 ± 3	9 ± 5
Capillaries per mm^2	1545 ± 572	2039 ± 613	1898 ± 595	1628 ± 324

Abbreviations: MSCs, mesenchymal stromal cells; MSCs-pVEGF, mesenchymal stromal cells transfected with pVEGF; pVEGF, plasmid encoding human vascular endothelial growth factor. No significant differences were found in either capillary or arteriolar densities at 30 days post treatment among groups. Mean ± s.d.

Importantly, the infarct-limiting effect occurred principally at the subendocardium, namely the myocardial zone displaying the most relevant role in LV mechanics.[20]

VEGF-overexpressing MSCs have been previously used in animal models of coronary artery disease. In rats, Matsumoto *et al.*[8] injected MSCs transduced with adenoviral-VEGF after 1 week of acute coronary occlusion. The treatment reduced infarct size and improved LV function, mainly due to increased capillary and arteriolar densities. Similar results were reported by Lu *et al.*,[13] who injected MSCs transduced with an adenovirus encoding VEGF in pigs 1 week after reperfused myocardial infarction via the coronary vein. Again, the proposed mechanisms were reduced collagen deposition and increased vasculogenesis. Apart from the species used and, in the case of Lu *et al.*,[13] the route of administration, the difference with our study is the vector used. Adenoviral vectors, although displaying higher transfection efficiency than plasmids, entail the risk of immune response. This makes them inadequate candidates for use in patients, especially if repeated administrations were eventually needed. On the other hand, plasmids, although exhibiting lower efficiency of transfection, are safe and amenable for repeated treatments, this facilitating clinical translation. By optimizing our transfection methods,[21] we attained 42.3 ± 4.7% transfection efficiency with near 80% cell viability, which sufficed to promote therapeutic effects.

Microvascular proliferation

Sheep receiving VEGF gene therapy and VEGF-overexpressing MSCs showed significantly increased density of capillaries. This angiogenic effect was to be expected on account that VEGF is a paradigmatic mitogen of endothelial cells. VEGF has also been shown to induce arteriolar proliferation.[22,23] In fact, in the present study—although not statistically significant—the group treated with direct injection of naked pVEGF had twice the arteriolar density of the placebo group. Furthermore, in *ex vivo* rat hearts, it has been shown that VEGF is a crucial mediator of the anti-ischemic protection afforded by MSCs.[24]

However, it was MSCs, either or not transfected with pVEGF, that promoted the highest arteriogenic response. It has been reported that MSCs induce arteriolar growth in animal models of ischemic heart disease,[25,26] an effect attributed to the paracrine action of released factors and signaling molecules.[27] In fact, the secretome of MSCs include growth factors known to induce migration and proliferation of smooth muscle cells, like fibroblast gowth factor-1, hepatocyte growth factor and platelet-derived growth factor.[28,29]

As MSCs often exert their beneficial effects through the release of paracrine factors, it has been pointed out that genetic modification can also contribute significantly to the MSC-mediated paracrine effects.[30] In fact, in our sheep, the group receiving VEGF-overexpressing MSCs displayed the strongest vasculogenic effect which, in turn, decreased infarct size.

It is interesting to note that in MSCs-pVEGF-treated animals the limit between viable myocardium and the infarct showed contractile tissue extending towards the scar, especially at the subendocardial level. This observation was consistent with the MR images, which showed that the decrease in infarct size was mainly due to subendocardial tissue salvage (see Figure 1). Given the predominant role of the subendocardium in systolic wall thickening and contractile performance,[20] it is not surprising that the improvement in %EF to almost its pre-IAM values was mainly due to a significant decrease of ESV with unchanged EDV.

Cell retention

An issue that remains unresolved in stem cell-based cardiac regeneration concerns cell retention in the myocardium.[31] It has been shown that the intramyocardial route yields significantly higher percent retention than the intracoronary and retrograde coronary venous routes.[32] However, already within few hours after intramyocardial delivery, cell retention is below 12%.[32] After 4 weeks of intramyocardial injection in pigs with AMI, it has been shown that only 0.35 ± 0.05% donor cells can be detected in the target tissue.[33] Consistent with these studies, and in agreement with the previously reported difficulties associated with cell retention, our cell tracking experiments showed that the injected VEGF-transfected MSCs, although readily visible at 7 days, were hardly detectable at 1 month after delivery.

Treatment dose and timing

One of the earliest studies in sheep and, to the best of our knowledge, the only one reporting a dose–response curve, compared 25, 75, 225 and 450×10^6 cells injected in the peri-infarct of sheep with AMI.[34] The authors found that the most significant effect on infarct size limitation and improved cardiac function was achieved with the lower doses (25 and 75×10^6 cells), and this was consistent with microvascular densities in the peri-infarct zone. Our animals received 20×10^6 cells, an amount numerically lower than those reported as maximally active by Hamamoto *et al.*,[34] but qualitatively better in terms of angiogenic potential, on account that they had been modified to overexpress VEGF.

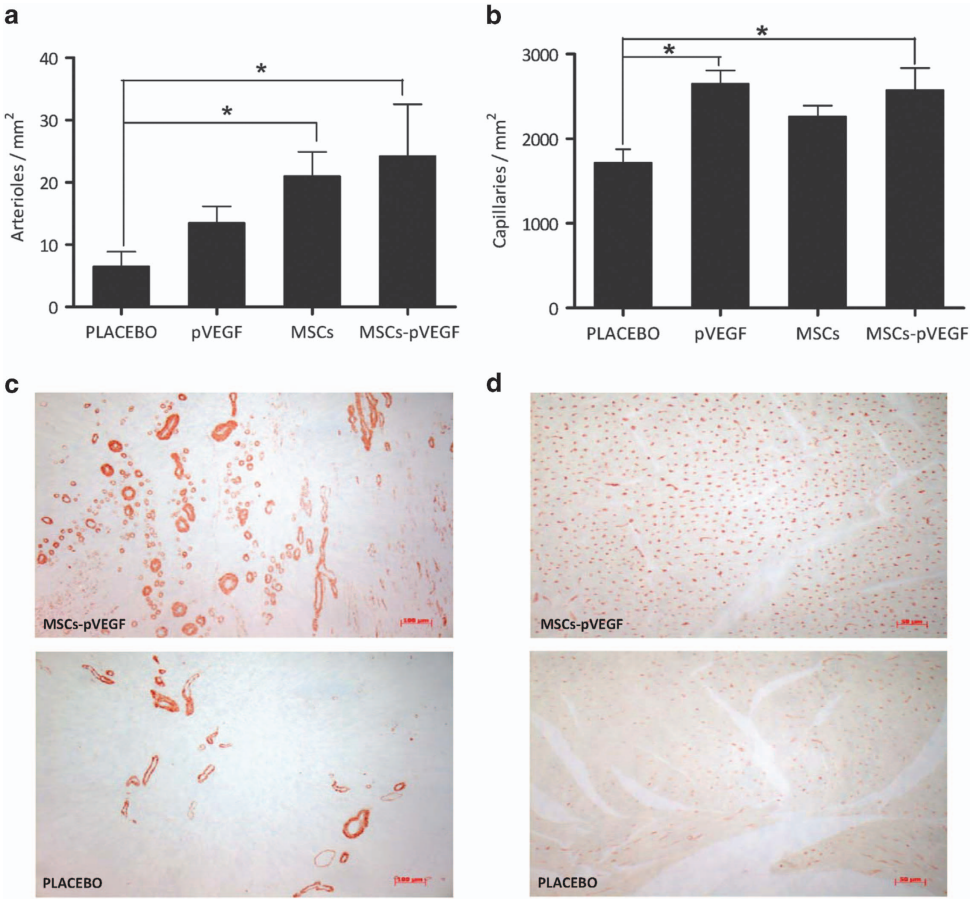

Figure 3. Microvascular proliferation. (**a**) At the end of follow-up, arteriolar density was significantly higher than placebo in the groups receiving VEGF-transfected MSCs and non-transfected MSCs. (**b**) Capillary density was significantly higher than placebo in the group receiving the naked plasmid-VEGF (pVEGF) and in the group treated with VEGF-transfected MSCs. (**c**) Arterioles (smooth muscle actin immunohistochemistry) in the peri-infarct zone of a sheep treated with VEGF-transfected MSCs (upper image) and placebo (lower image). Bars: 100 μm. (**d**) Peri-infarct capillaries (Euonymus Europaeus Lectin immunohistochemistry) in a MSCs-pVEGF-treated sheep (upper image) and a placebo-treated sheep (lower image). Bars: 50 μm. $^{*}P < 0.01$. Mean ± s.d.

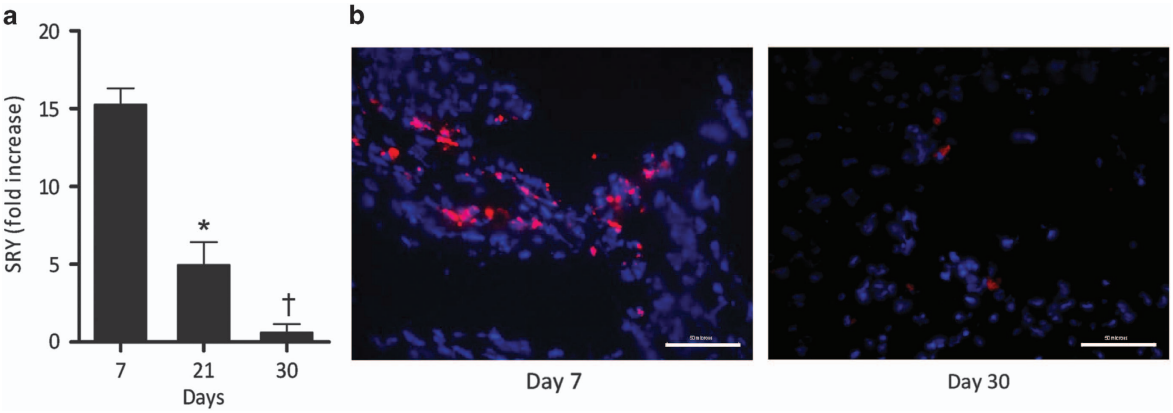

Figure 4. Cell tracking and transgene expression. (**a**) Detection (qPCR) of the sex-determining SRY gene in the myocardium injected with VEGF-transfected MSCs at 7, 21 and 30 days post injection. Values are mean ± s.d. of three measurements. (**b**) VEGF-transfected MSCs marked with the red fluorescent dye PKH26 in the peri-infarct myocardium at 7 and 30 days post injection. Nuclei are stained blue (DAPI). Bars: 50 μm. Values are mean ± s.d. $^{*}P < 0.05$ vs 7 days; $^{\dagger}P < 0.05$ vs 7 and 21 days.

With regard to the group receiving pVEGF, the dose (3.8 mg) was selected on the basis of our previous results. Both in sheep with myocardial infarction and pigs with chronic myocardial ischemia, 3.8 mg of the plasmid induced significant angiogenesis and arteriolar proliferation, attenuating fibrosis and enhancing LV function.[16–19,22] Moreover, a recent phase I clinical study in patients with severe ischemic heart disease showed that the dose was safe and reduced significantly the ischemic burden of the LV as assessed by single photon emission computed tomography under pharmacological stress.[35] In the present study, 3.8 mg

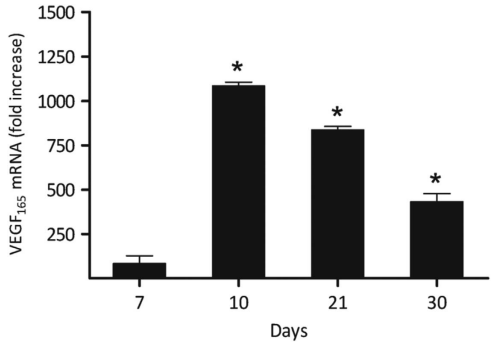

Figure 5. Human VEGF mRNA detection (RT-qPCR) in the myocardium injected with VEGF-transfected MSCs at 7, 10, 21 and 30 days post injection. Values are mean ± s.d. of three measurements. $^*P < 0.05$ vs 7 days.

pVEGF reduced infarct size, though to a lesser extent than transfected MSCs, and tended to improve LV %EF. We can not rule out that higher doses would have enhanced these cardioprotective effects. However, 3.8 mg is, to the best of our knowledge, the highest pVEGF dose used so far in large animal and human studies of plasmid-VEGF-mediated angiogenesis for ischemic heart disease.

As concerns treatment timing, it has been repeatedly shown that after AMI, the window for myocardial tissue salvage based on direct reperfusion approaches is narrow. Current guidelines recommend a door-to-balloon time of 90–120 min.[36] However, cardioprotective strategies based on stem cell transplant have been shown to be more effective at later time points after AMI.[37–39] We decided to treat our sheep at 1 week after coronary ligature on the basis of previous results from other authors. Hu *et al.*[37] correlated the therapeutic benefit of MSCs in rat AMI with time of administration. They showed that maximal infarct size reduction, attenuation of LV chamber dilation and improvement of cardiac function was attained when the cells were injected 7 days after AMI, a time point in which scar formation has not yet occurred and inflammation is reduced, as has also been described for rabbit AMI.[38] Moreover, in a pooled analysis of 660 patients included in randomized controlled trials addressing safety and efficacy of intracoronary bone marrow stem cells, Zhang *et al.*[39] showed that transfer at 4–7 days post AMI was significantly superior in terms of improved LV %EF and reduced LV end-systolic dimensions than within the first 24 h.

In addition, for a potential use in the clinical setting, the risk of complications derived from puncturing the myocardium would be lower than at earlier time points.

Assessment of infarct size and LV function

Given the heterogeneity of the left anterior descending artery distribution in sheep,[40] it was necessary to assess for infarct size on a paired analysis basis. We therefore carried out MRI within the first 72 h after coronary occlusion and at the end of the follow-up. MRI has been shown to be the most appropriate imaging method for investigating ischemic heart disease. In AMI, MRI allows to quantify and characterize the pattern of ischemic myocardial damage in terms of area at risk and infarct size, and thereby of salvaged myocardium, along with depicting microvascular damage.[41]

As regards LV function, we have previously shown that echocardiographic parameters of systolic and diastolic LV performance in young adult sheep can be reliably extrapolated to the adult human,[42] this indicating that at least in this animal model, echocardiography is useful to assess LV function.

Study limitations

We used young adult sheep in our study because in terms of biological features such as life span, gestation time and heart size, sheep are closer than laboratory rodents to the human, this allowing for a more reliable extrapolation of results to the clinic. However, the model does not display the risk factors and comorbidities present in human ischemic heart disease, which have been shown to limit the angiogenic capacity of diverse stem cells.[43–45] In fact, this might explain, at least in part, the failure of clinical trials to reproduce the promising results obtained in animal experiments. Similar concerns apply to age. While our sheep are young, aged patients are predominant in ischemic heart disease. On account that MSCs harvested from elderly patients are weaker in terms of growth and therapeutic effect on myocardial infarction than those from young donors,[46] genetically modifying MSCs to overexpress VEGF may be especially useful. In effect, VEGF-overexpressing (but not naïve) MSCs from old rats have been shown to increase capillary density, preserve cardiac function and reduce infarct size similarly than non-transfected MSCs obtained from young animals.[11]

Another limitation of the model is the scarcity of commercially available antibodies that react with ovine antigens. This problem may challenge the correct characterization of ovine MSCs. In our case, we had to restrict the surface antigen analysis to the reaction against CD166, CD44 and CD45 antibodies. However, we confirmed the mesenchymal phenotype of the cells through their ability to differentiate into adipocytes, chondrocytes and osteocytes.

The delivery route used in the present study has been shown to be the best in terms of cell retention. However, a problem inherent to the intramyocardial route is that a part of the injectate is expelled back by systolic contraction, which determines that the amount of cells effectively delivered is less than planned. We attenuated this drawback by keeping the needle in place for 10–15 s after each injection, but could not prevent it completely.

Finally, the follow-up time of our protocol prevents us from knowing whether the therapeutic effect lasts in the long term and, if so, for how long.

CONCLUSION

We conclude that in sheep with AMI, the injection of allogeneic VEGF-overexpressing MSCs reduces infarct size and restores LV systolic function to a larger extent than non-transfected MSCs or the naked plasmid-VEGF, by promoting capillary and arteriolar proliferation. Myocardial salvage occurs predominantly at the subendocardium, this enhancing the recuperation of contractile performance. Given the ease of obtention and growth in culture of MSCs, their potential for allogeneic use and the safety associated with the use of a plasmid vector, this strategy may prove potentially applicable in the clinical setting.

MATERIALS AND METHODS

All procedures were carried out in accordance with the Guide for Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996) and approved by the Laboratory Animal Care and Use Committee (CICUAL) of the Favaloro University.

MSCs obtention and processing

The methods used for MSCs extraction, characterization and transfection have been reported.[21] Briefly, 10–15 ml bone marrow was collected from the iliac crest of male donor sheep under deep sedation. Samples were processed with Ficoll-Hypaque (1.077 g ml^{-1}) density gradient and the mononuclear cell layer was isolated. Cells were plated and incubated at 37 °C and 5% CO_2 with Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with antibiotic-antimycotic (Anti-Anti, Gibco),

20% (v/v) fetal bovine serum (Internegocios S.A., Buenos Aires, Argentina), and 2 mmol glutamine (Gibco). Cells were cultured until 80–90% confluence. Passages 3–5 were used.

Cell surface antigen expression was assessed by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA) using human anti-CD166 (BD Biosciences), ovine anti-CD44 (AbD Serotec, Oxford, UK) and ovine anti-CD45 (AbD Serotec) antibodies (Figure 6). Cells were positive for CD166 and CD44, and negative for CD45. Further immune typification was not feasible given the scarcity of commercially available antibodies for sheep antigens. In addition, cells were successfully differentiated *in vitro* into adipocytes (Red Oil, Sigma Aldrich, St Louis, MO, USA), osteocytes (Alizarin Red, Sigma-Aldrich) and chondrocytes (Alcian Blue 8 GX, Sigma-Aldrich) using *ad-hoc* kits (Gibco) (Figure 7).

To genetically modify MSCs, we tested varying amounts of transfection reagents combined with growing masses of a plasmid encoding green fluorescent protein (pCruz GFP, Santa Cruz Biotechnology, Santa Cruz, CA, USA). By combining Lipofectamine LTX (Gibco) 5 µl and Plus reagent (Gibco) 4 µl with 2 µg DNA, we obtained the maximal transfection efficiency (42.3 ± 4.7%) with a cell survival of 78.9%.[21] Using these transfection conditions, we transfected MSCs with a plasmid encoding human $VEGF_{165}$ (pVEGF, Biosidus, Buenos Aires, Argentina). The VEGF protein concentration in the culture supernatant was measured (ELISA) at day 0 (184 ± 21.8 pg ml^{-1} per µg DNA), day 2 (1968 ± 324 pg ml^{-1} per µg DNA), day 6 (1200.1 ± 150.8) and day 12 (888 ± 386 pg ml^{-1} per µg DNA) post transfection. Moreover, the supernatant displayed an intense tubulogenic effect in a tube formation assay.[21]

Surgical preparation and treatment

Thirty-two Corriedale sheep weighing 36.8 ± 4.5 Kg were premedicated with acepromazine maleate 5 mg intramuscular. Anesthesia was induced with propofol 3 mg kg^{-1} intravenous and maintained with 2% isoflurane in oxygen under mechanical ventilation (Neumovent, Córdoba, Argentina). A sterile thoracotomy was performed at the fourth intercostal space, the pericardium was opened and an anterior-apical infarct comprising approximately 15% of the LV mass was induced by coronary artery ligation. Given that sheep display inter-individual variability in the left anterior descending artery distribution, the branches to be ligated were selected by visual inspection of the epicardial left anterior descending anatomy.[38] To reduce the incidence of ventricular arrhythmias, we administered lidocaine (three bolus injections of 2 mg each and a 2 mg kg^{-1} h^{-1} infusion), amiodarone (150 mg infusion in 2 h) and atenolol (2 mg). Subsequently, the thoracotomy was repaired without pericardial closure and, after removal of the tracheal tube, cephalotin 1 g intravenous was injected, and sheep were returned to the animal house under analgesic treatment.

One week after AMI, sheep were reoperated and randomized into four treatment groups ($n = 8$ per group): phosphate-buffered saline (placebo group), $pVEGF_{165}$ 3.8 mg (pVEGF group), 2×10^7 MSCs (MSCs group) or 2×10^7 pVEGF-transfected MSCs (MSCs-pVEGF group). All treatments, diluted in a final volume of 2 ml phosphate-buffered saline were delivered by direct intramyocardial transepicardial injection in the peri-infarct area, divided into 10 aliquots of 200 µl each. The nature of the injectates was kept blind for all investigators involved in data analysis and processing.

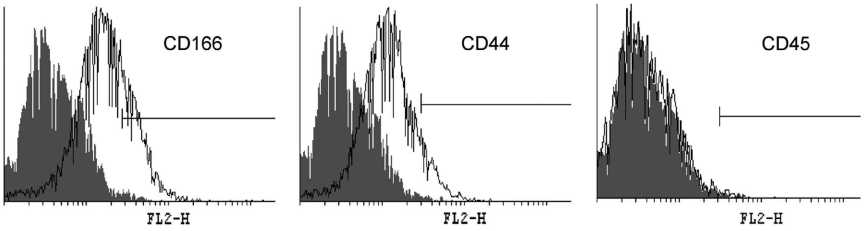

Figure 6. Ovine MSCs characterization by flow cytometry. Cells were positive for the surface antigens CD166 and CD44, and negative for CD45. (Reproduced from Locatelli *et al.*,[21] with permission from Elsevier).

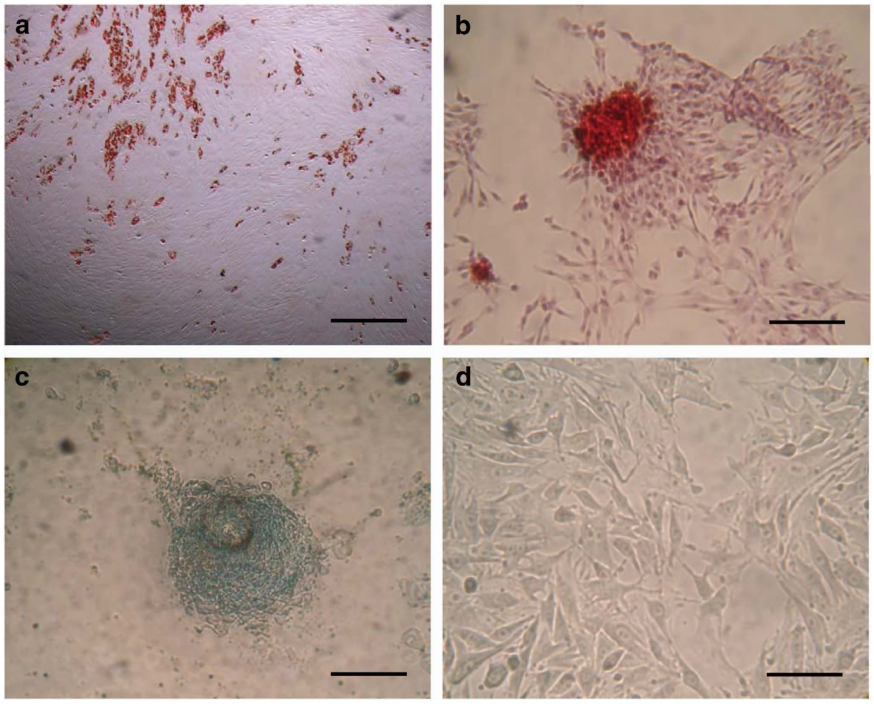

Figure 7. Differentiation of ovine MSCs into the adipocyte (**a**), osteocyte (**b**) and chondrocyte (**c**) lineages. (**d**) Control. Bars: 50 µm.

Infarct size

To allow for a paired analysis of infarct size, MRI (Philips Achieva 1.5 Tesla high field, Amsterdam, the Netherlands) was performed 3 days after infarction and 30 days after treatment.

Under deep sedation, 8–9 short-axis slices (8-mm thick) scanning the left ventricle were obtained. For infarct size quantification, the technique of delayed enhancement was used. A bolus of intravenous gadolinium contrast (0.1–0.2 mmol kg^{-1}) was followed 20 min later by cardiac image acquisition. The contours of the gadolinium-enhanced (necrotic) and non-enhanced (normoperfused) areas were delineated, and by use of the Segment software for quantitative medical image analysis (V1.8 R1172 Medviso AB, Lund, Sweden), infarct size was calculated as percent necrosis of total LV mass.

LV function

At 3 days after AMI and at 30 days post treatment, bidimensional echocardiography was performed under light sedation (diazepam 10 mg intravenous) and with the animal lying on its right lateral decubitus (Sonos 5500, Hewlett-Packard, Palo Alto, CA, USA) using 2.5 to 4 MHz transducers. Parasternal long- and short-axis views were recorded. EDV and ESV calculated by single-plane multiple overlapped disks method and LV %EF were measured. It should be noted that biplane methods could not be applied because in sheep, it is not possible to obtain truly orthogonal views from the parasternal window.

Hemodynamics

Prior to killing of the sheep, LV catheterization was performed using a pressure tip catheter (Millar MikroTip, Millar Instruments, Houston, TX, USA) under deep sedation with propofol. The transducer control unit (TC-510, Millar) was connected to an amplifier (Gould 2400S, Gould Electronics, Cleveland, OH, USA), a monitor (Sirecust 404-1, Siemens, Erlangen, Germany) and a computer with the aid of an A/D expansion card. After calibration, the catheter was advanced to the LV via the right carotid artery and the intraventricular pressure signal (frequency: 250 Hz) was acquired. Heart rate, peak systolic pressure, end-diastolic pressure and the maximum 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 10 s acquisition time. At the end of the procedure, the animal was killed with an overdose of propofol followed by a bolus injection of potassium chloride to arrest the heart in diastole.

Additional sheep for short-term analysis

Sixteen additional sheep ($n=4$ animals per group) were operated to induce AMI, re-operated and treated following the protocol explained above, and killed as stated above at 10 days after treatment. Samples were collected for histological and molecular analysis. For histological analysis (microvascular density and fibrosis), three transmural myocardial samples, each encompassing infarcted tissue, the infarct border and the peri-infarct zone were harvested, fixed in 10% folmaldehyde and embedded in paraffin. For detection of the VEGF mRNA, one transmural sample of the peri-infarct (injected) zone was collected and stored at −80 °C.

Tissue sections were deparaffinized and brought to phosphate-buffered saline solution, pH 7.2. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol, antigens were retrieved with citrate buffer pre-treatment in a microwave oven and the slides were incubated during 1 h with a specific monoclonal antibody against smooth muscle actin (BioGenex, Fremont, CA, USA) to identify arterioles (vessels measuring 8–100 μm in diameter displaying a smooth muscle media layer), the endothelial marker Biotinylated Euonymous Europaeus Lectin (Vector Laboratories, Burlingame, CA, USA) to identify capillaries (vessels with only an endothelial wall measuring up to 10 μm in diameter) and an anti-Ki67 antibody (BioGenex) to detect cardiomyocyte nuclei undergoing the cell cycle. Twenty matching fields (×10 magnification for arteriole and ×20 for capillary quantification) from each slide were examined (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD, USA). The scanned area was $283 \pm 73\ mm^2$ for the placebo group, $269 \pm 47\ mm^2$ for pVEGF, $255 \pm 77\ mm^2$ for MSCs and $282 \pm 76\ mm^2$ for MSCs-pVEGF. Vascular density was expressed as number of capillaries and arterioles per mm^2 and cardiomyocyte mitotic index as number of Ki67-positive nuclei per million cardiomyocyte nuclei. Additional transmural sections were stained with Masson's trichrome to analyze the limit between the viable myocardium and the fibrotic scar. Fibrosis was expressed as the percentage of blue-stained areas within an area of 2 mm to each side of a line perpendicular to the epicardial limit of the infarction.

Additional sheep for cell tracking and VEGF expression analysis

To assess for cell retention, AMI was induced in three additional female sheep using the surgical procedure described above. A total of 2×10^7 VEGF-transfected MSCs from a male donor were marked with the red fluorescent dye PKH26 (Sigma-Aldrich) and injected in 10 sites of the peri-infarct zone. Sheep were killed as described above at 7 ($n=1$), 21 ($n=1$) and 30 ($n=1$) days after treatment. Myocardial samples of the infarct border zone were harvested and stored at −80 °C. DAPI-stained cryosections of the animals killed at 7 and 30 days were examined under fluorescence microscopy to detect VEGF-transfected MSCs. On samples from all three sheep, the presence of the SRY (sex determining region-Y) gene of the donor male was assessed by qPCR.

For gene expression analysis, frozen myocardial tissue from sheep of the four experimental groups was pulverized and homogenized in Trizol reagent (Gibco) according to the manufacturer's instructions. Expression of human $VEGF_{165}$ was assessed by real-time qPCR. The threshold cycle (Ct) values were determined and normalized to GAPDH to adjust for equal amounts of RNA. Forward and reverse primers used in this assay were (SRY: Upper (forward) primer: 5′CAG ACA ATC ATA GCG CAA ACG 3′; Lower (reverse) primer: 5′GCT GCT CTC CCT AAC ATT TTC C 3′. VEGF: Upper (forward) primer: 5′ ACG TAC TTG CAG ATG TGA CAA G 3′; Lower (reverse) primer: 5′ GTG GCG GCC GCT CTA 3′).

Animal loss

To attain 51 useful sheep (32 corresponding to the 30 days follow-up protocol, 16 to the 10 days follow-up protocol and 3 corresponding to cell tracking experiments), 57 animals were operated. The six lost animals (11.1%) died of irreversible ventricular fibrillation during surgery or in the early postoperative period.

Statistical analysis

Data were analyzed using one-way or two-way analysis of variance followed by Bonferroni tests using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Results are expressed as mean ± s.d. Statistical differences were considered significant at $P < 0.05$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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