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ORIGINAL ARTICLE

Allogeneic adipose stem cell therapy in acute myocardial infarction

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ABSTRACT

Background Stem cell therapy offers a promising approach to reduce the long-term mortality rate associated with heart failure after acute myocardial infarction (AMI). To date, *in vivo* translational studies have not yet fully studied the immune response to allogeneic adipose tissue-derived mesenchymal stem cells (ATMSCs). We analysed the immune response and the histological and functional effects of allogeneic ATMSCs in a porcine model of reperfused AMI and determine the effect of administration timing.

Design Pigs that survived AMI (24/26) received intracoronary administration of culture medium after reperfusion ($n = 6$), ATMSCs after reperfusion ($n = 6$), culture medium 7 days after AMI ($n = 6$) or ATMSCs 7 days after AMI ($n = 6$). At 3-week follow-up, cardiac function, alloantibodies and histological analysis were evaluated.

Results Administration of ATMSCs after reperfusion and 7 days after AMI resulted in similar rates of cell engraftment; some of those cells expressed endothelial, smooth muscle and cardiomyogenic cell lineage markers. Delivery of ATMSCs after reperfusion compared with that performed at 7 days was more effective in increasing: vascular density (249 ± 64 vs. 161 ± 37 vessels/mm2; $P < 0{\cdot}01$), T lymphocytes (1 ± 0·4 vs. 0·4 ± 0·3% of area $CD3^+$; $P < 0{\cdot}05$) and expression of vascular endothelial growth factor (VEGF; 32 ± 7% vs. 20 ± 4% of area $VEGF^+$; $P < 0{\cdot}01$). Allogeneic ATMSC-based therapy did not change ejection fraction but generated alloantibodies.

Conclusions The present study is the first to demonstrate that allogeneic ATMSCs elicit an immune response and, when administered immediately after reperfusion, are more effective in increasing VEGF expression and neovascularization.

Keywords Adipose tissue-derived stem cells, allogeneic stem cells, experimental study, myocardial infarction, neovascularisation, time of administration.

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Introduction

Reperfusion therapy and concomitant medical treatment have reduced short-term mortality after acute myocardial infarction (AMI), but a large percentage of patients still develop negative remodelling with reduced ventricular function leading to chronic heart failure [1]. Currently, the only treatment for end-stage chronic heart failure is heart transplantation, but this is limited due to the lack of organ donation and the comorbidities that make patients ineligible. Therefore, development of new therapeutic approaches to prevent end-stage heart failure following AMI is of great interest, which has led to basic research in recent years on the possible use of stem cells for this purpose.

Among all sources of stem cells, multipotent cells can also be obtained from the adipose tissue. Adipose tissue-derived mesenchymal stem cells (ATMSCs) are available in large amounts, expand easily and rapidly *in vitro* and can differentiate into multiple tissue lineages [2,3]. Furthermore, experimental studies have demonstrated in small animal models that engrafted ATMSCs expressed endothelial and cardiac markers [4,5], and our group has shown in a porcine model of AMI that engrafted autologous ATMSCs were incorporated into newly formed vessels and enhanced neovascularisation [6].

[1]Both authors have contributed equally to this study.

Although basic research is still needed to address the mechanisms governing cardiac repair using autologous stem cells, the use of allogeneic stem cells may be a promising clinical alternative to preparing autologous cells from the recipient as allogeneic cells would be immediately available when needed. Moreover, allogeneic cells would be obtained from young and healthy donors, avoiding the use of cells from older patients suffering from coronary artery disease with reduced levels and functional impairment of progenitor cells [7,8].

Current *in vitro* studies have demonstrated that allogeneic bone marrow mesenchymal stem cells (BMMSCs) have several properties that contribute to the inhibition of T cell proliferation and thus avoid rejection [9]; on the other hand, *in vivo* studies observed that these stem cells induce a complete immune response [10–12]. To date, *in vivo* translational studies have not yet fully studied the immune response to allogeneic ATMSCs.

Changes in the microenvironment following AMI can affect transplanted stem cell homing, survival, engraftment and differentiation [13]. However, few experimental studies have investigated the best moment for stem cell administration and to our knowledge no studies have investigated in large animals if ATMSCs could produce different effects when ATMSCs are administered at different times after reperfused AMI.

Therefore, the aim of the present research was to analyse the immune response and the histological and functional effects of allogeneic ATMSCs in a porcine model of reperfused AMI and determine whether the time of administration affected outcomes.

Materials and methods

Experimental animals

The management of the 32 pigs (26 recipients and 6 donors) used conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (Publication No. 86–23, revised 1996). Experiments with animals and histological analysis were performed between February 2009 and May 2010 and from May 2010 to December 2011, respectively.

Twenty-four (32 ± 2 kg) of the 26 animals that survived initial induced AMI received: group 1, intracoronary culture medium 15 min after coronary reperfusion (n = 6); group 2, intracoronary ATMSCs 15 min after coronary reperfusion (n = 6); group 3, intracoronary culture medium 7 days after AMI (n = 6); and group 4, intracoronary ATMSCs 7 days after AMI (n = 6). Pigs were followed up 3 weeks after ATMSCs or culture medium administration (Figure S1 and Data S1).

Adipose tissue collection and cell labelling

Donor animals (n = 6) were anesthetised (Figure S1 and Data S1), and subcutaneous adipose tissue was acquired to isolate and characterize mesenchymal stem cells as described previously [6]. Cells were expanded and kept at −80 °C until further use. Prior to *in vivo* administration, frozen aliquots were thawed and some of these ATMSCs (2×10^6cells) were labelled by transfection with a gene encoding β-Gal [6] (Figure S1 and Data S1). One inguinal lymph node was excised to determine donor-specific antibodies formation in sera of recipient animals.

AMI procedure

Recipient animals were anaesthetized as previously described [6], and cardiac function was assessed by intracardiac echocardiogram (AcuNav Catheter, Biosense, Diamond Bar, CA, USA). Left ventricular end-diastolic (LVEDV) and systolic (LVESV) volumes and ejection fraction (LVEF) were measured using the Simpson method. AMI was then induced, as previously described [14]. Briefly, an angioplasty catheter was introduced into the left anterior descending artery, and the balloon was inflated for 90 min, followed by reperfusion.

Intracoronary administration of ATMSCs

On the same day of AMI (15 min after reperfusion) or 7 days after AMI, cardiac function was again measured by echocardiogram and culture medium, or allogeneic ATMSCs were intracoronary administered [6] (Figure S1 and Data S1). There were no significant differences between groups in total ATMSCs administered (10 ± 0 millions in group 2 vs. 13 ± 4 millions in group 4).

Harvesting of specimens

After 3-week follow-up, echocardiographic analysis was performed to determine cardiac function and serum was obtained to detect alloantibodies in recipient animals. The hearts were excised, cut into transversal slices and prepared for histological analysis [6] (Figure S1 and Data S1).

Samples of kidney, lung, intestine, spleen, liver and tracheobronchial nodes involved in the lymphatic drainage of the heart were also harvested to determine the presence of β-galactosidase $(\beta\text{-Gal})^+$ cells.

Infarct size

To analyse the percentage of left ventricle infarction, the necrotic zone of each transversal slice was measured using morphometric analysis [6] (Figure S1 and Data S1).

Cellular engraftment

β-Gal staining (Roche Diagnostics, Sant Cugat del Vallès, Barcelona, Spain) was performed to evaluate ATMSCs grafted in the myocardium and in the studied organs. $\beta\text{-Gal}^+$ cells (visible by the blue colour) were expressed as counts per cm^2 [6].

Cellular phenotype

Double fluorescent immunohistochemistries were perform to study ATMSCs differentiation into cardiomyocytes, smooth muscle cells or endothelial cells, using anti-β-Gal antibody (Abcam, Cambridge, UK) and antitroponin I (TnI) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-smooth muscle actin (SMA) antibody (Abcam) or anti-von Willebrand Factor (vWF) antibody (Dako, Glostrup, Denmark) [6] (Figure S1 and Data S1).

Double-positive cells were blindly evaluated under a confocal microscope (Leica Microsystems, Wetzlar, Germany) by two independent investigators at ×630 magnification.

Vascular endothelial growth factor (VEGF) expression

For each animal, five sections from the infarct border zone were immunostained with anti-VEGF antibody (Santa Cruz Biotechnology) to identify VEGF expression (Figure S1 and Data S1).

Five areas of each section were examined under light microscopy at ×100 magnification and blindly analysed (AnalySIS, Olympus Optical, Hamburg, Germany). The results were expressed as a percentage of the area occupied by $VEGF^+$ cells in the infarct border zone.

Vascular density and myofibroblasts

For each animal, five sections were used to perform double immunohistochemistry with anti-SMA and anti-vWF antibodies [6] (Figure S1 and Data S1).

Five areas of each section were analysed under light microscopy at ×200 magnification and blindly analysed. Small vessels (15 μm or less in diameter) stained (vWF^+ cells) or double stained (SMA^+ and vWF^+ cells) were counted and expressed as vessels per mm^2.

Myofibroblasts (SMA^+ cells) with morphological features like fibroblasts were scored semiquantitatively in the infarct border zone. The analysis was based on visual estimation of slides at ×100 magnification; each section was assigned a score of low [1], intermediate [2] or high [3].

Presence of $CD3^+$ cells

For each animal, five sections from the infarct border zone were immunostained with rabbit anti-CD3 antibody (Dako) to identify T lymphocytes [15] (Figure S1 and Data S1).

Five areas of each section were examined under light microscopy at ×100 magnification and blindly analysed. The results were expressed as a percentage of the area occupied by $CD3^+$ cells in the infarct border zone.

Donor-specific antibodies formation

The presence of donor-specific antibodies 3 weeks after ATMSCs administration was tested using an adaptation of the standard flow cytometry crossmatch on donor lymph node cells, used for human solid organ transplantation as previously described [15] (Figure S1 and Data S1).

Statistical analysis

Quantitative variables are presented as mean ± SD values or median [quartiles 1–3] when criteria for normal distribution of data were not met. Student's unpaired *t*-test was used to compare quantitative variables between two groups and Mann–Whitney *U*-test when data were not normally distributed. ANOVA and Tukey's HSD *post hoc* procedures were used to compare quantitative variables between the four study groups and Kruskal–Wallis and Mann–Whitney *U*-test when criteria for normal distribution of data were not met. A $P < 0{\cdot}05$ was considered significant. Calculations were performed using the SPSS/PC statistical package (SPSS Inc., Chicago, IL, USA).

Results

During coronary occlusion procedure, two pigs died as a consequence of ventricular fibrillation (8% mortality).

Left ventricular function

Table 1 shows sequence changes in LVEDV, LVESV and LVEF from baseline, with different administration times and 3 weeks after treatment. There were no significant differences between the groups (ANOVA, P = NS).

After AMI, LVESV increased and, as a consequence, LVEF was reduced vs. baseline values in all groups, although reduction was greater in groups 3 and 4 because AMI developed 7 days before measurement.

In all the groups, 3 weeks after culture medium or ATMSCs administration, there was no improvement in LVEF over values after AMI.

Infarct size

The percentage of infarcted left ventricle was 24 ± 4%, 21 ± 4%, 21 ± 3% and 25 ± 3% for groups 1, 2, 3 and 4, respectively, without any differences between groups (ANOVA, P = NS).

ATMSCs engraftment and phenotype

Three weeks after ATMSCs administration, there were no significant differences in the number of engrafted labelled cells ($\beta\text{-gal}^+$ cells) between animals treated with ATMSCs 15 min after coronary reperfusion (group 2) and animals treated with ATMSCs 7 days after AMI (group 4; 6·25 [3·7–9·6] vs. 13·5 [2·5–15·6] labelled cells/cm^2, respectively; P = NS). In addition, engrafted $\beta\text{-Gal}^+$ cells, 92 ± 8% (group 2) and 86 ± 12% (group 4; P = NS) were localized in the infarct border zone and close to vessels. No engrafted $\beta\text{-Gal}^+$ cells were found in remote organs.

Table 1 Left ventricular volumes and ejection fraction measured by echocardiography

	Ad 15 min after reperfusion		Ad 7 days after AMI	
	CM (control) $n = 6$	ATMSCs $n = 6$	CM (control) $n = 6$	ATMSCs $n = 6$
Baseline				
LVEF (%)	77 ± 4	80 ± 5	80 ± 6	78 ± 6
LVEDV (mL)	35·6 ± 7·5	37·8 ± 13·6	24·9 ± 10·2	22·5 ± 6·7
LVESV (mL)	8·5 ± 3	7·7 ± 4·1	5·2 ± 2·9	4·7 ± 2
After reperfusion				
LVEF (%)	59 ± 13	60 ± 9*	–	–
LVEDV (mL)	36·8 ± 7·5	29·9 ± 12·2	–	–
LVESV (mL)	15·1 ± 6·5	12·6 ± 4·5	–	–
7 days after AMI				
LVEF (%)	–	–	52 ± 3*	54 ± 4*
LVEDV (mL)	–	–	30·4 ± 20·4	20·5 ± 9·3
LVESV (mL)	–	–	14·3 ± 9·3	9·4 ± 4·1
3 weeks after treatment				
LVEF (%)	51 ± 9*	53 ± 3*	52 ± 7*	52 ± 10*
LVEDV (mL)	46·3 ± 5	34·5 ± 12·7	30·8 ± 22·7	23·7 ± 6·2
LVESV (mL)	22·3 ± 3·9*	16·1 ± 6·3*	15·5 ± 11·8	10·9 ± 4·5

Results presented as mean ± SD.
CM, culture medium; Ad, administration; AMI, acute myocardial infarction; ATMSCs, adipose tissue-derived mesenchymal stem cells; LVEDV, left ventricular end-diastolic volumes; LVEF, left ventricular ejection fraction; LVESV, left ventricular end-systolic volumes.
*$P < 0·05$ vs. baseline.

Confocal microscopy analysis evidenced β-Gal$^+$ cells additionally expressing TnI, SMA and vWF in group 2 and in group 4, suggesting implanted cell differentiation into cardiomyogenic, vascular smooth muscle and endothelial lineage (Fig. 1).

VEGF expression

The results of VEGF expression evaluated as percentage of area occupied by VEGF$^+$ cells in the infarct border zone are shown in Fig. 2a–e. VEGF expression was similar in both treatment groups (1 and 3) that received culture medium regardless of time of administration (13·8 ± 5·2 vs. 13·6 ± 3·3%, respectively; P = NS), whereas VEGF expression was higher when ATMSCs were given 15 min after reperfusion (group 2) compared with administration 7 days after AMI (group 4; 31·6 ± 7·1 vs. 20·4 ± 3·7%, respectively; $P < 0·01$). It is also important to note that VEGF expression was significantly greater in the group that received ATMSCs (group 2) compared with the group that received culture medium (group 1) 15 min after AMI (31·6 ± 7·1 vs. 13·8 ± 5·2%, respectively; $P < 0·001$). However, these differences did not reach statistical significance when comparing groups receiving ATMSCs (group 4) or culture medium (group 3) 7 days after AMI (20·4 ± 3·7 vs. 13·6 ± 3·3%, respectively; P = NS).

Vascular density and myofibroblasts

There were no significant differences in the number of vessels in the infarct border zone between groups receiving culture medium (groups 1 and 3; 171·3 ± 34·7 vs. 179·4 ± 44 vessels/mm^2, respectively; P = NS). However, animals treated with ATMSCs 15 min after coronary reperfusion (group 2) showed higher number of vessels in the infarct border zone than animals treated with ATMSCs 7 days after AMI (group 4; 249 ± 63·9 vs. 161·1 ± 37·1 vessels/mm^2, respectively; $P < 0·01$). Moreover, comparison between groups receiving culture medium and ATMSCs resulted in significant differences when were administered 15 min after AMI (groups 1 vs. 2; 171·3 ± 34·7 vs. 249 ± 63·9 vessels/mm^2, respectively; $P < 0·01$) but not 7 days after AMI (groups 3 vs. 4; 179·4 ± 44 vs. 161·1 ± 37·1 vessels/mm2, respectively; P = NS; Fig. 3a–e).

Semiquantitative analysis of myofibroblasts presence in the infarct border zone showed more myofibroblasts with ATMSCs administration 15 min after reperfusion (group 2) com-

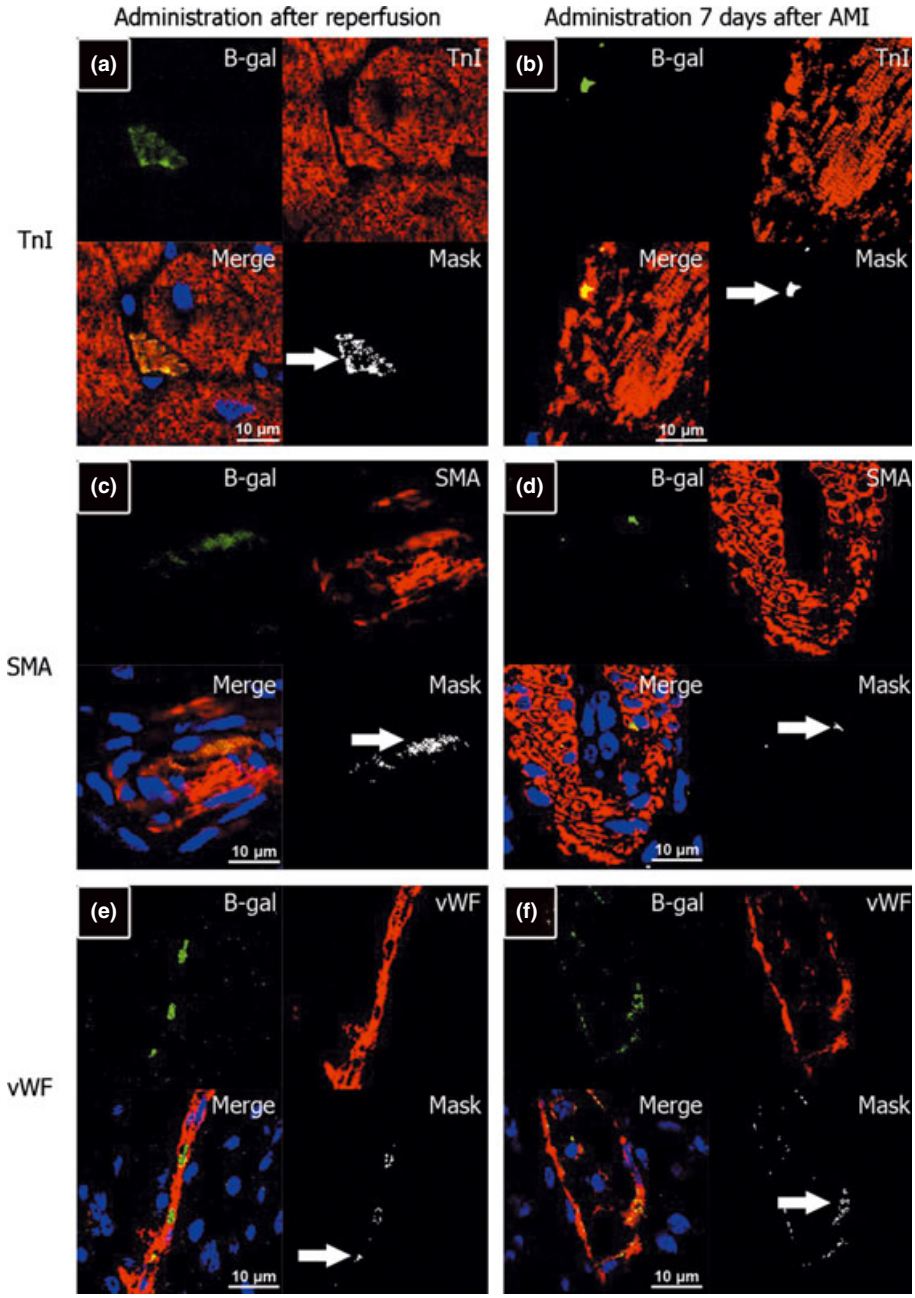

Figure 1 Different phenotypes of adipose tissue-derived mesenchymal stem cells (ATMSCs) in the infarct border zone, 3 weeks after their administration. Confocal microscopy images showing colocalization of β-Gal$^+$ cells staining for troponin I (TnI) (a and b), alpha smooth muscle actin (SMA) (c and d) or Von Willebrand factor (vWF) (e and f). Arrows indicate double-positive cells. Merge = merged image; Mask = colocalization of red and green colours (white colour). Percentage of green colocalized with red in particular cells indicated by arrows was: 91% (a), 99% (b), 94% (c), 64% (d), 74% (e) and 94% (f).

pared with the other groups (1, 3 and 4; score per group: 3 [2–3] vs. 2 [1–2], 2 [1–2] and 2 [1–2], respectively; Fig. 3f–j).

Presence of CD3$^+$ cells

The different time of culture medium administration did not modify the percentage of area occupied by CD3$^+$ cells (lymphocytes T) in the infarct border zone (0·37 ± 0·2 vs. 0·36 ± 0·22, respectively; P = NS). However, the percentage of area occupied by CD3$^+$ cells was significantly greater in animals treated with ATMSCs 15 min after coronary reperfusion (group 2) compared with those treated with ATMSCs 7 days after AMI (group 4; 1·04 ± 0·41 vs. 0·45 ± 0·31%, respectively; $P < 0·05$). Furthermore, animals treated 15 min after AMI with ATMSCs (group 2) showed higher area occupied by CD3$^+$ cells than animals receiving culture medium (group 1; 1·04 ± 0·41 vs. 0·37 ± 0·2, respectively; $P < 0·05$). However, there were no significant differences in the area occupied by CD3 + cells between groups receiving culture

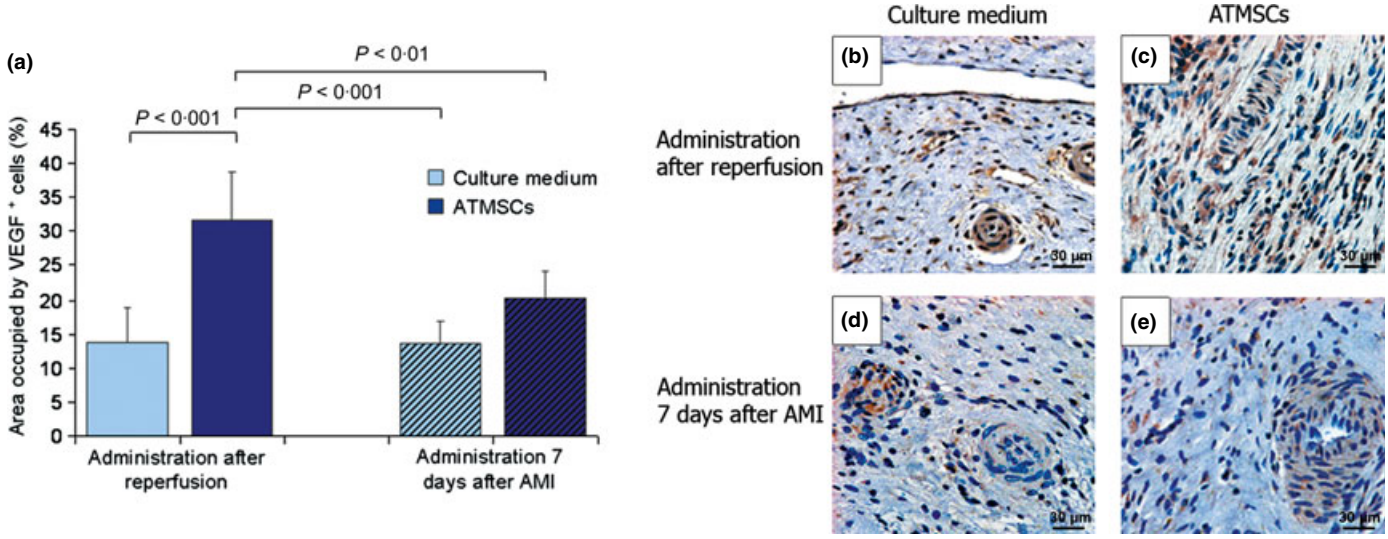

Figure 2 Vascular endothelial growth factor (VEGF) expression in the infarct border zone. (a) Percentage of area occupied by VEGF$^+$ cells (stained in brown). (b–e) Representative images showing higher expression of VEGF in the group of animals treated with ATMSCs 15 min after reperfusion (c) than in the other groups (b, d and e).

medium (group 3) and ATMSCs (group 4) 7 days after AMI (0·36 ± 0·22 vs. 0·45 ± 0·31%, respectively; P = NS; Fig. 4).

Donor-specific antibodies formation

At 3-week follow-up, donor-specific antibodies formation was found in the serum of 2 of the 6 animals (33·3%) in both groups (2 and 4) treated with ATMSCs.

Discussion

This is the first study to analyse the immune response to allogeneic ATMSCs administered at different times in a porcine model that reproduces the pathophysiological characteristics of reperfused AMI. The main finding of our study is that allogeneic ATMSCs are not entirely immunoprivileged after their intracoronary administration and elicit an immune response characterized by donor-specific antibodies formation and presence of CD3$^+$ cells, with more positive CD3 cells when ATMSCs are administered after myocardial reperfusion. However, the injection of ATMSCs 15 min after reperfusion was more effective in increasing the expression of vascular endothelial growth factor (VEGF), neovascularisation and myofibroblasts that could positively affect long-term prognosis.

Donor-specific antibodies formation and presence of CD3$^+$ cells

We examined allogeneic cells because, although admittedly they could induce rejection, their advantage is that functional stem cells would be ready and available to be administered when needed by patients undergoing primary coronary angioplasty. In fact, we found at 3-week follow-up, donor-specific antibodies formation occurred in 33·3% of animals treated with ATMSCs, a rate of allosensitization similar to that found in human tissue and porcine arterial transplantation [16,17]. To the best of our knowledge, very few studies have specifically investigated the immune response to allogeneic mesenchymal stem cells. Mainly, there were studies conducted in small animals. Those studies performed in large animals with AMI administered BMMSCs, while our study utilized ATMSCs. Poncelet *et al.* [10] and Huang *et al.* [12] administered allogeneic BMMSCs after AMI and found that although these allogeneic mesenchymal stem cells had a promising low immunogenic profile *in vitro*, when transplanted *in vivo* they initiated an immunogenic phenotype and triggered a complete immune response. Our histological analysis confirmed that some engrafted allogeneic ATMSCs expressed endothelial cells, smooth muscle cells and cardiomyocytes markers, as reported in previous studies with allogeneic adipose tissue-derived stem cells in rodents [4,18]. ATMSCs differentiation may explain why these cells were not immunoprivileged *in vivo* and induced immune rejection after administration as previously described in mesenchymal stem cells [19].

Although allogeneic ATMSCs are immune rejected, this obvious limitation is not an important problem if most of the allogeneic ATMSCs were deleted from cardiac tissue at least 3 weeks after their intracoronary administration. As suggested by Hashemi *et al.* [20] with BMMSCs and our results in ATMSCs, most of the benefits observed with the administration of

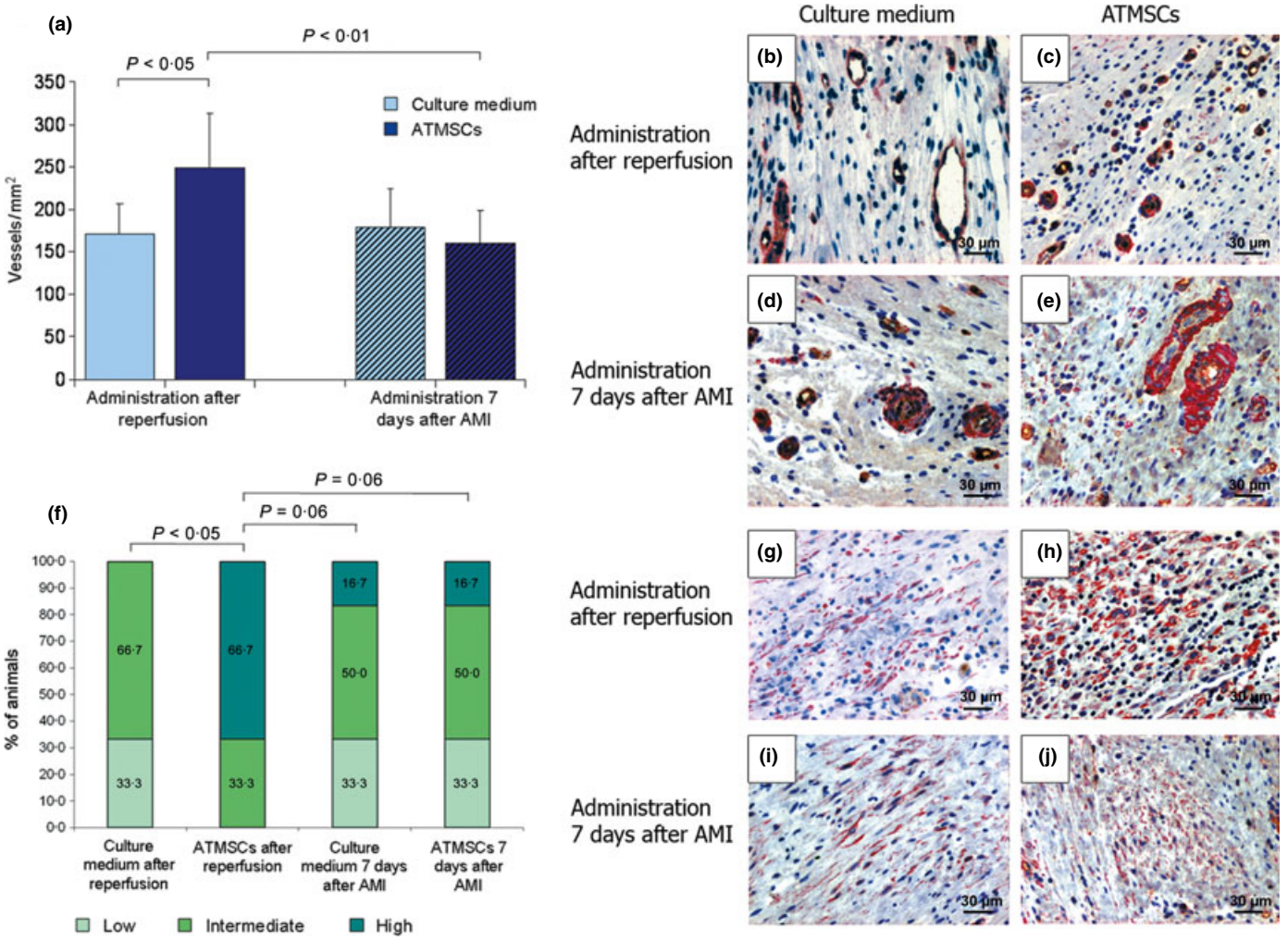

Figure 3 Vascular density and myofibroblasts presence. (a) The number of vessels per mm^2 in the infarct border zone for study groups. (b–e) Microphotographs showing the comparative results of vascular density in the infarct border zone for all the groups. The number of vessels (smooth muscle cells stained in red and endothelial cells stained in brown) was higher after administration of ATMSCs 15 min after reperfusion. (f) Percentage of animals with low, intermediate and high presence of myofibroblasts in the infarct border zone in each study group. (g–j) Representative images of the myofibroblasts presence (stained in red) in the border zone. The group of animals treated with ATMSCs 15 min after reperfusion (h) shows a higher presence of myofibroblasts than other groups (g, i and j).

allogeneic mesenchymal stem cells appear to be produced by an indirect paracrine effect and not by a large number of implanted and differentiated cells. Therefore, allogeneic ATMSCs would act soon after administration and, after transient residence with relevant beneficial effects, be eliminated along with the adverse effects of rejection.

Time of administration

We have demonstrated, for the first time, better neovascularization and greater number of myofibroblasts when allogeneic ATMSCs were given just after myocardial reperfusion than a week later. Our histological analysis showed that one mechanism by which allogeneic ATMSCs increased neovascularisation is VEGF production. Some studies have also demonstrated that resident and transplanted stem cells exert beneficial effects on damaged tissue through a paracrine mechanism by releasing factors, such as SDF1, HGF, TGFβ1 and VEGF, involved in cell homing, tissue fibrosis and neovascularisation [3,21]. Moreover, myofibroblasts isolated from the site of AMI were derived from endothelial and smooth muscle cells in response to TGFβ1

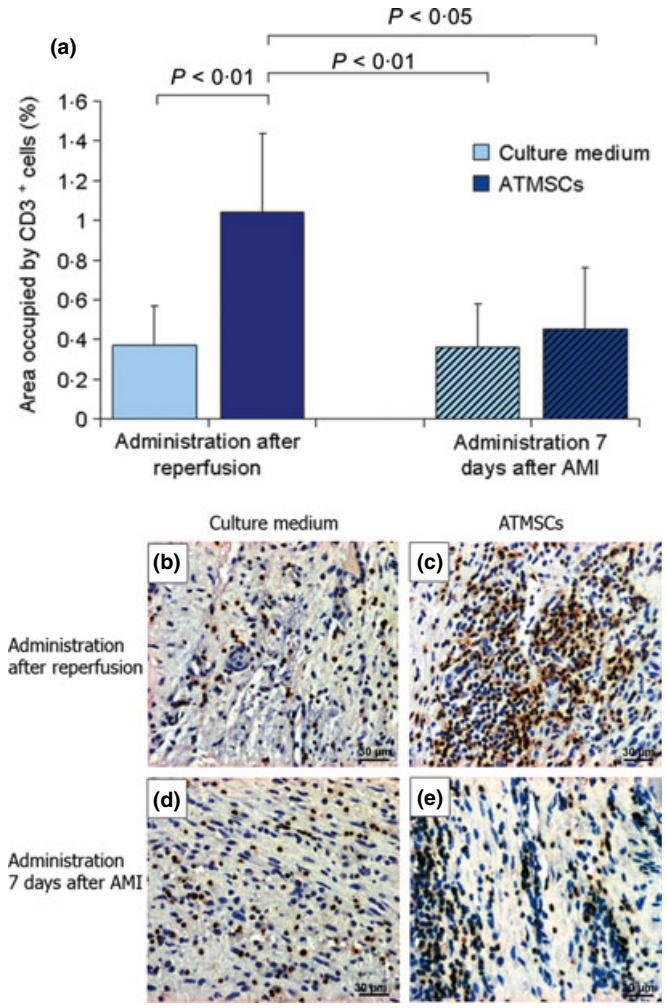

Figure 4 Lymphocytes T present in the infarct border zone. (a) Percentage of area occupied by $CD3^+$ cells (stained in brown). (b–e) Representative images showing higher presence of lymphocytes T in the group of animals treated with ATMSCs 15 min after reperfusion (c) than in the other groups (b, d and e).

and produce angiogenic factors such as VEGF [22,23]. However, our study design precludes evaluation of this possibility.

Both groups of animals treated with ATMSCs resulted in similar rates of engrafted cells at 3-week follow-up. However, we also observed more $CD3^+$ cells when allogeneic ATMSCs were administered after myocardial reperfusion. Although speculative, this may be due partly to a more reactive moment (ischaemia/reperfusion damage) and partly to more early allogeneic ATMSCs homing. Immune response would act over time deleting allogeneic cells, and thus, there were low rates of engrafted cells in both groups 3 weeks after ATMSCs administration. Therefore, these very low rates of engraftment made it difficult to detect significant differences between groups at 3-week follow-up. To date, few experimental and clinical studies have addressed the optimal time of stem cell administration [24–27], and this issue is still unsettled but very relevant for clinical practice. During the first hours and days after myocardial reperfusion, restoration of coronary blood flow is accompanied by generation of reactive oxygen species, invasion of leucocytes and release of signalling factors and cytokines. This acute inflammatory reaction progressively decreases until the week after myocardial reperfusion when subacute granulation phase begins [13,28]. This myocardial microenvironment facilitates stem cell actions through the participation of some cytokines, such as stromal cell-derived factor-1 and hepatocyte growth factor, related to stem cell homing and survival of administered and resident stem cells, as reported by Ma *et al.* and O'Blenes *et al.* in rodents [24,29] and also suggested by our results. A recent study, similar to ours, by Kupatt *et al.* [30] in a preclinical pig model of AMI with ischaemia/reperfusion found that the retroinfusion of adult endothelial progenitor cells immediately after reperfusion exerted cardioprotective effects by reducing infarct size and improving regional myocardial function.

In our study, the greater number of new vessels and myofibroblasts found in animals receiving allogeneic ATMSCs after reperfusion did not result in significant LEVF improvement at 3-week follow-up. However, in the long term, new vessels found in the infarct border zone could translate into better regional perfusion and remodelling [31,32], and new myofibroblasts, which may persist for longer, could prevent later dilatation of the infarct area through the maintenance of the extracellular matrix [22]. Although no other published studies in large animals or humans have used allogeneic ATMSCs, results of an experimental study of intracoronary administration of allogeneic BMMSCs were comparable to ours in terms of ejection fraction [33].

Limitations

It cannot be ruled out that ischaemia/reperfusion damage could have played a role in the increase in $CD3^+$ cells found when ATMSCS were administrated after myocardial reperfusion. Moreover, the study design precludes reporting on potential long-term benefits of ATMSCs therapy. Determining whether the initial beneficial effects translate into reverse remodelling will require another study.

Conclusion

In summary, the present study is the first to investigate the immune response to allogeneic ATMSCs administered at two time points after AMI in a relevant animal model of ischaemia/reperfusion. Porcine allogeneic ATMSCs elicited an immune response after their intracoronary administration, differentiated

into both cardiomyocytes and vascular cells, administration immediately after coronary reperfusion more effectively increased expression of VEGF expression and neovascularisation. Future studies are required to investigate the effects on LVEF in a long-term survival model.

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Contributions

M. Rigol is responsible for planning and design of study experiments, subcutaneous adipose tissue collection, infarct procedure, intracoronary administration of cells, echo images analysis, histological studies, and data analysis and interpretation. He is the cowriter of the manuscript. N. Solanes is responsible for planning and design of study experiments, subcutaneous adipose tissue collection, infarct procedure, intracoronary administration of cells, histological studies, and data analysis and interpretation. He is the cowriter of the manuscript. S Roura is responsible for isolation and expansion of subcutaneous adipose-derived mesenchymal stem cells, and culture experiments and transfection of cells by nonviral technology. He is the cowriter of the manuscript. M. Roqué is responsible for co-induction of myocardial infarction and intracoronary administration of adipose-derived mesenchymal stem cells and reviewed the manuscript. L. Novensà is responsible for anaesthesia and animal postoperative care. A. Dantas involved in the standardization of the transfection method of cells by nonviral technology and reviewed the manuscript. J. Martorell involved in the determination of donor-specific antibodies formation and reviewed the manuscript. M. Sitges involved in the co-analysis of echo images and interpretation of the results and reviewed the manuscript. J. Ramírez involved in the interpretation of histological analysis and reviewed the manuscript. A Bayés-Genís involved in the planning and codesign of study experiments, data interpretation and critical review of the manuscript. M. Heras, Project coordinator, involved in the planning and codesign of study experiments, data interpretation and critical review of the manuscript.

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References

1 Sans S, Puigdefabregas A, Paluzie G, Monterde D, Balaguer-Vintro I. Increasing trends of acute myocardial infarction in Spain: the MONICA-Catalonia Study. *Eur Heart J* 2005;**26**:505–15.
2 Bai X, Alt E. Myocardial regeneration potential of adipose tissue-derived stem cells. *Biochem Biophys Res Commun* 2010;**401**:321–6.
3 Mazo M, Gavira JJ, Pelacho B, Prosper F. Adipose-derived stem cells for myocardial infarction. *J Cardiovasc Trans Res* 2011;**4**:145–53.
4 Bai X, Yan Y, Song Y, Seidensticker M, Rabinovich B, Metzele R *et al.* Both cultured and freshly isolated adipose tissue-derived stem cells enhance cardiac function after acute myocardial infarction. *Eur Heart J* 2010;**31**:489–501.
5 Bayes-Genis A, Soler-Botija C, Farré J, Sepúlveda P, Raya A, Roura S *et al.* Human progenitor cells derived from cardiac adipose tissue ameliorate myocardial infarction in rodents. *J Mol Cell Cardiol* 2010;**49**:771–80.
6 Rigol M, Solanes N, Farré J, Roura S, Roqué M, Berruezo A *et al.* Effects of adipose tissue-derived stem cell therapy after myocardial infarction: impact of the route of administration. *J Cardiac Fail* 2010;**16**:357–66.

7 Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H *et al.* Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001;**89**:e1–7.
8 Rauscher FM, Goldschmidt-Clermont PJ, Davis BH, Wang T, Gregg D, Ramaswami P *et al.* Aging, progenitor cell exhaustion and atherosclerosis. *Circulation* 2003;**108**:457–63.
9 Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res* 2004;**95**:9–20.
10 Poncelet AJ, Vercruysse J, Saliez A, Gianello P. Although pig allogeneic mesenchymal stem cells are not immunogenic in vitro, intracardiac injection elicits an immune response in vivo. *Transplantation* 2007;**83**:783–90.
11 Poncelet AJ, Hiel AL, Vercruysse J, Hermans D, Zech F, Gianello P. Intracardiac allogeneic mesenchymal stem cell transplantation elicits neo-angiogenesis in a fully immunocompetent ischaemic swine model. *Eur J Cardiothorac Surg* 2010;**38**:781–7.
12 Huang XP, Sun Z, Miyagi Y, McDonald Kinkaid H, Zhang L, Weisel RD *et al.* Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. *Circulation* 2010;**122**:2419–29.
13 Horst KW. Stem cell therapy for myocardial infarction: are we missing time? *Cardiology* 2010;**117**:1–10.
14 Rigol M, Solanes N, Roqué M, Farré J, Batlle M, Roura S *et al.* Haemosiderin deposits confounds tracking of iron-oxide labelled stem cells. *Transpl Proc* 2008a;**40**:3619–22.
15 Solanes N, Rigol M, Khabiri E, Castellà M, Ramírez J, Roqué M *et al.* Effects of cryopreservation on the immunogenicity of porcine arterial allografts in early stages of transplant vasculopathy. *Cryobiology* 2005;**51**:130–41.
16 Rigol M, Solanes N, Sionis A, Gálvez C, Martorell J, Rojo I *et al.* Effects of cyclosporine, tacrolimus and sirolimus on vascular changes related to immune response. *J Heart Lung Transplant* 2008b;**27**:416–22.
17 Smith JD, Hornick PI, Rasmi N, Rose ML, Yacoub MH. Effect of HLA mismatching and antibody status on "homovital" aortic valve homograft performance. *Ann Thorac Surg* 1998;**66**:S212–5.
18 Léobon B, Roncalli J, Joffre C, Mazo M, Boisson M, Barreau C *et al.* Adipose-derived cardiomyogenic cells: in vitro expansion and functional improvement in a mouse model of myocardial infarction. *Cardiovasc Res* 2009;**83**:757–67.
19 Buja LM, Vela D. Immunologic and inflammatory reactions to exogenous stem cells. Implications for experimental studies and clinical trials for myocardial repair. *J Am Coll Cardiol* 2010;**56**:1693–700.
20 Hashemi SM, Ghods S, Kolodgie FD, Parcham-Azad K, Keane M, Hamamdzic D *et al.* A placebo controlled, dose-ranging, safety study of allogenic mesenchymal stem cells injected by endomyocardial delivery after an acute myocardial infarction. *Eur Heart J* 2008;**29**:251–9.
21 Tang JM, Wang JN, Zhang L, Zheng F, Yang JY, Kong X *et al.* VEGF/SDF-1 promotes cardiac stem cell mobilization and myocardial repair in the infarcted heart. *Cardiovasc Res* 2011;**91**:402–11.
22 Van den Borne SWM, Diez J, Balnkesteijn WM, Verjans J, Hofstra L, Narula J. Myocardial remodelling after infarction: the role of myofibroblasts. *Nat Rev Cardiol* 2010;**7**:30–7.
23 Chintalgattu V, Harris GS, Akula Sm, Katwa LC. PPAR-γ agonists induce the expression of VEGF and its receptors in cultured cardiac myofibroblasts. *Cardiovasc Res* 2007;**74**:140–50.
24 Ma J, Ge J, Zhang S, Sun A, Shen J, Chen L *et al.* Time curse of myocardial stromal cell-derived factor 1 expression and beneficial effects of intravenously administered bone marrow stem cells in rats with experimental myocardial infarction. *Basic Res Cardiol* 2005;**100**:217–23.
25 Hu X, Wang J, Chen J, Luo R, He A, Xie X *et al.* Optimal temporal delivery of bone marrow mesenchymal stem cells in rats with myocardial infarction. *Eur J Cardiothorac Surg* 2007;**31**:438–43.
26 Li RK, Mickle DA, Weisel RD, Rao V, Jia ZQ. Optimal time for cardiomyocyte transplantation to maximize myocardial function after left ventricular injury. *Ann Thorac Surg* 2001;**72**:1957–63.
27 Sürder D, Manka R, Lo Cicero V, Moccetti T, Rufibach K, Soncin S *et al.* Intracoronary injection of bone marrow-derived mononuclear cells early or late after acute myocardial infarction. *Circulation* 2013;**127**:1968–79.
28 Bartunek J, Wijns W, Heyndrickx GR, Vanderheyden M. Timing of intracoronary bone-marrow-derived stem cell transplantation after ST-elevation myocardial infarction. *Nat Clin Pract Cardiovasc Med* 2006;**3**:S52–6.
29 O'Blenes SB, Li AW, Chen R, Arora RC, Horackova M. Engraftment is optimal when myoblasts are transplanted early: the role of hepatocyte growth factor. *Ann Thorac Surg* 2010;**89**:829–35.
30 Kupatt C, Hinkel R, Lamparter M, von Brühl ML, Pohl T, Horstkotte J *et al.* Retroinfusion of embryonic endothelial progenitor cells attenuates ischemia-reperfusion injury in pigs. *Circulation* 2005;**112**:I117–22.
31 Mazo M, Hernández S, Gavira JJ, Abizanda G, Araña M, López-Martínez T *et al.* Treatment of reperfused ischemia with adipose-derived stem cells in a preclinical swine model of myocardial infarction. *Cell Transplant* 2012;**21**:2723–33.
32 Ling L, Shen Y, Wang K, Jiang Ch, Fang Ch, Ferro A *et al.* Worse clinical outcomes in acute myocardial infarction patients with type 2 diabetes mellitus: relevance to impaired endothelial progenitor cells mobilization. *PLoS One* 2012;**7**:e50739.
33 Dubois C, Liu X, Claus P, Marsboom G, Pokreisz P, Vandenwijngaert S *et al.* Differential effects of progenitor cell population on left ventricular remodelling and myocardial neovascularization after myocardial infarction. *J Am Coll Cardiol* 2010;**55**:2232–43.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. The flow-chart depicts the study design.

