Intracoronary Delivery of Self-Assembling Heart-Derived Microtissues (Cardiospheres) for Prevention of Adverse Remodeling in a Pig Model of Convalescent Myocardial Infarction

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Background—Preclinical studies in rodents and pigs indicate that the self-assembling microtissues known as cardiospheres may be more effective than dispersed cardiosphere-derived cells. However, the more desirable intracoronary route has been assumed to be unsafe for cardiosphere delivery: Cardiospheres are large (30–150 μm), raising concerns about likely microembolization. We questioned these negative assumptions by evaluating the safety and efficacy of optimized intracoronary delivery of cardiospheres in a porcine model of convalescent myocardial infarction.

Methods and Results—First, we standardized the size of cardiospheres by modifying culture conditions. Then, dosage was determined by infusing escalating doses of cardiospheres in the left anterior descending artery of naïve pigs, looking for acute adverse effects. Finally, in a randomized efficacy study, 14 minipigs received allogeneic cardiospheres (1.3×10⁶) or vehicle 1 month after myocardial infarction. Animals underwent magnetic resonance imaging before infusion and 1 month later to assess left ventricular ejection fraction, scar mass, and viable mass. In the dosing study, we did not observe any evidence of microembolization after cardiosphere infusion. In the post-myocardial infarction study, cardiospheres preserved LV function, reduced scar mass and increased viable mass, whereas placebo did not. Moreover, cardiospheres decreased collagen content, and increased vessel densities and myocardial perfusion. Importantly, intracoronary cardiospheres decreased left ventricular end-diastolic pressure and increased cardiac output.

Conclusions—Intracoronary delivery of cardiospheres is safe. Intracoronary cardiospheres are also remarkably effective in decreasing scar, halting adverse remodeling, increasing myocardial perfusion, and improving hemodynamic status after myocardial infarction in pigs. Thus, cardiospheres may be viable therapeutic candidates for intracoronary infusion in selected myocardial disorders. (Circ Cardiovasc Interv. 2015;8:e002391. DOI: 10.1161/CIRCINTERVENTIONS.115.002391.)

Key Words: myocardial infarction  ■ stem cells

Ischemic heart disease is the leading cause of death in the world, accounting for at least 7 million deaths per year.¹² New treatments to improve outcome after myocardial infarction (MI) are desirable. Heart-derived cell products have particular promise in this regard.³ The first described human cardiac stem cell products were self-assembling multicellular three-dimensional (3D) microtissues, which Messina et al⁴ dubbed cardiospheres. These microtissues had diameters of ≈30 to >200 μm; when injected intramyocardially, cardiospheres improved function and structure in a mouse model of acute MI. Given that capillaries have diameters of only ≈8 μm, intracoronary delivery of cardiospheres was assumed to be implausible given the likelihood of coronary microembolization.⁵⁶ In contemplating translation to the clinic, we preferred to use the intracoronary route whose safety had been well-validated in previous clinical trials of cell therapy. Thus, we developed cardiosphere-derived cells (CDCs) as a dispersed single-cell product grown from replated cardiospheres. CDCs delivered intracoronary turned out to work well, both in animal models and in humans,⁷⁻¹¹ but we were left wondering whether cardiospheres might have worked even better. When compared head-to-head in small- or large-animal MI models using intramyocardial delivery, cardiospheres were at least as effective as CDCs, and often more so. Therefore, in this study, we questioned our negative
Assumptions about the viability of intracoronary cardiosphere infusion. We reasoned that perhaps, if cardiosphere sizes were standardized and dose/delivery were carefully optimized, we might be able to infuse cardiospheres safely down a coronary artery. We initially optimized cardiosphere size using different culture conditions, and performed a dose-ranging study in minipigs to assess the feasibility and safety of intracoronary cardiosphere infusion. Finally, we performed a blinded, prospective placebo-controlled study in a porcine model of convalescent MI to assess the safety and efficacy of intracoronary cardiosphere infusion.

**Methods**

Animal studies were performed in an American Association for Accreditation of Laboratory Animal Care accredited facility with approval from the Cedars-Sinai Institutional Animal Care and Use Committee (detailed Methods are available in the Data Supplement).

**Growth of Cardiospheres and Optimization of Culture Conditions**

CDCs were grown from a freshly explanted heart obtained from a male Sinclair minipig, and secondary cardiospheres were made from CDCs at the fifth passage using cardiosphere media as described.4,12 Culture time and cell-seeding density were optimized to obtain a minimal percentage of cardiospheres >150 μm (which may cause severe microvascular obstruction) and a high percentage of cardiospheres >50 μm (to avoid the infusion of single cells). For this purpose, we compared 5 different seeding densities and 4 different harvest time points (Figure 1A). Size, number, and distribution of the cardiospheres obtained were determined using a particle counter. The cardiospheres generated (from 12M plated CDCs) were counted before and after ex vivo passage through a microcatheter (Finecross, Terumo, Tokyo, Japan) to quantify retention of cardiospheres in the catheter.

**WHAT IS KNOWN**

- Heart-derived stem cell clusters (cardiospheres) improve post myocardial infarction heart function and structure when injected intramuscularly, but are assumed to be too large for safe intracoronary delivery.
- Cardiosphere-derived cells are effective and safe when infused via the coronary route in animals and in humans.
- Cardiospheres are equally or more efficacious than cardiosphere-derived cells when both are injected intramuscularly in animals.

**WHAT THE STUDY ADDS**

- Optimization of cardiosphere manufacturing methods consistently yields particles of 50 to 100 μm size.
- Dose- and size-optimized cardiospheres can be safely delivered via the coronary route, increasing function and decreasing adverse remodeling in a pig model of convalescent myocardial infarction.
- Intracoronary delivery of cardiospheres produces intense angiogenic effects and may be even more effective than intracoronary cardiosphere-derived cells.

![Figure 1. Schematic representation of the in vivo study (A): 5 different seeding densities and 4 different times for harvest were used to optimize cardiosphere size. Increasing cell density increases the average size of cardiosphere (B), decreases the proportion of small cardiospheres (<50 μm) (C1) and increases the proportion of 50 to 100 μm cardiospheres (C2). In contrast, increasing culture duration increases the number of cardiospheres in the 100 to 150 μm range (C3) and cardiospheres >150 μm (C4). The conditions chosen for the in vivo studies are those giving high numbers of cardiospheres >50 μm with few cardiospheres >150 μm (labeled with red arrow in B and C1–C4). Images of small, midsize and large cardiospheres are shown in (D); nuclei are stained with 4′,6-diamidino-2-phenylindole (blue) and cell membranes are stained with calcein acetomethoxy (green). CDC indicates cardiosphere-derived cells.](http://circinterventions.ahajournals.org/doi/abs/10.1161/CIRCINTERVENTIONS.117.008887)
For in vivo studies, secondary cardiospheres from 12.5M CDCs were frozen and thawed the day of infusion. Viability of the thawed cardiospheres was assessed using homoe-thidium bromide (which stains dead nuclei red).

**Study Design**

Two separate experimental protocols were performed as depicted in Figure 2. Briefly, a study was first performed to determine the maximal feasible dose and, in a second step, a blinded placebo-controlled study was performed to assess efficacy of infused cardiospheres. A total of 26 Yucatan minipigs were used: 7 completed the dose study and 14 completed the efficacy study. Three pigs died, 2 after MI creation, and 1 after placebo infusion. Two pigs were excluded because of technical issues during MI creation (1 balloon deflation leading to inadequate MI, and 1 left anterior descending artery dissection).

**MI Creation and Cardiosphere Infusion**

For the feasibility study, increasing doses of cardiospheres were administered in naive Yucatan minipigs. The doses were defined by the number of single cells used to manufacture the cardiospheres (single-cell equivalent [SCE]). Pigs were infused with 12.5x10^6, 25x10^6, and 50x10^6 SCE, corresponding to ≈3.25x10^5, 6.5x10^5, and 1.3x10^6 multicellular particles, respectively (Figure I in the Data Supplement). All intracoronary infusions were performed using continuous flow (no flow interruption during infusion, no balloon inflation), with a microcatheter (Finecross) placed in the mid–left anterior descending artery. Safety was assessed by adverse events during infusion (eg, arrhythmias, dissection, and hypotension), Thrombolysis In Myocardial Infarction (TIMI) flow after infusion, left ventricular ejection fraction (LVEF) measured by LV gram before and after infusion (to detect potential myocardial stunning related to microembolization), and troponin I increase 24 hours after infusion.

For the efficacy study, MIs were created in female adult Yucatan minipigs by inflating an angioplasty balloon in the mid–left anterior descending artery just after the first diagonal branch for 2.5 hours. For the efficacy study, MIs were created in female adult Yucatan minipigs by inflating an angioplasty balloon in the mid–left anterior descending artery just after the first diagonal branch for 2.5 hours. Three weeks later, animals were randomized to receive cardiospheres (50x10^6 SCE, 1.3x10^6 particles) or vehicle using continuous flow infusion. Safety was assessed as in the dose study. LV end-diastolic pressure (LVEDP) was recorded using a pigtail catheter placed into the LV cavity. Left ventriculography was then performed to assess LV function. Minipigs were euthanized 1 month after infusion. All procedures and analyses were performed blinded to treatment allocation.

**Magnetic Resonance Imaging**

Magnetic resonance imaging was performed on a 3.0 Tesla MRI scanner (Siemens Magnetom Verio, Erlangen, Germany) at baseline (3 weeks after MI, before infusion) and 1 month post infusion.

**Coronary Flow Reserve Measurement**

Coronary flow reserve was quantified with a Doppler wire as described in the Data Supplement.

**Histology**

Histological analyses were performed on 8-μm sections from myocardial samples (fixed in 10% formalin and embedded in paraffin) obtained from infarct and border zone (3–6 slides per heart) and remote zone (2 slides per heart).

**Statistical Analysis**

Continuous variables are presented as mean±SD in the text and mean±SE in the figures. Categorical variables are expressed as absolute number and percentage. Independent groups (cardiospheres and placebo) were compared using Mann–Whitney U test. Wilcoxon test was used to compare paired groups (changes from baseline). All P values are 2 sided, and a P<0.05 was considered statistically significant.

**Results**

**Cardiosphere Culture Optimization**

Figure 1B shows a histogram of cardiosphere size distribution at various times and seeding densities. Increased seeding...
density is associated with a higher proportion of spheres >50 μm (Figure 1C), whereas longer culture time mostly increased the percentage of big particles (>150 μm; Figure 1D). Therefore, a high-seeding density (1.5×10^6 CDCs in 75 cm² flasks, ie, 20×10^3 CDCs/cm²) and short culture duration (48 hours) were deemed optimal for cardiospheres manufacturing. The number and size of secondary cardiospheres manufactured from 12.5M CDCs using these conditions are shown in Figure I in the Data Supplement.

Using optimally manufactured cardiospheres, we observed 5±9% total retention of cardiospheres in the catheter and ≈10% retention for cardiospheres >50 μm (Figure 1C), whereas longer culture time mostly increased the percentage of big particles (>150 μm; Figure 1D). Therefore, a high-seeding density (1.5×10^6 CDCs in 75 cm² flasks, ie, 20×10^3 CDCs/cm²) and short culture duration (48 hours) were deemed optimal for cardiospheres manufacturing. The number and size of secondary cardiospheres manufactured from 12.5M CDCs using these conditions are shown in Figure I in the Data Supplement.

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Safety of Cardiosphere Infusion

A dose escalation study was performed in 7 naïve animals to assess feasibility and safety. Pigs were infused with 12.5×10^6 SCE (n=2), 25×10^6 SCE (n=3), and 50×10^6 SCE (n=2). No arrhythmia occurred during cardiosphere infusion and no impairments of TIMI flow or LVEF after infusion were observed (Figure 3A and 3B). Troponin increase 24 hours after infusion was low without any difference among the 3 doses (Figure 3C).

To further assess safety, the heart of a pig infused with 50M SCE and euthanized 24 hours after infusion was stained with tetrazolium chloride. No myocardial necrosis was evident, either in the infused region or in remote areas (Figure II in the Data Supplement). Therefore, the highest dose (50M SCE) was used for further studies.

Safety was then confirmed in infarcted animals. After infusion of cardiospheres (dose of 50M SCE), we observed no
impairment of LVEF when compared with vehicle infusion; also, troponin I and creatine kinase at 24 hours were similar in cardiosphere and placebo groups (Figure 3D–3G). No impairment in TIMI flow was observed after vehicle infusion, whereas it decreased from TIMI 3 to TIMI 2 in 1 of 7 pigs infused with cardiospheres. This decrease in TIMI flow was most likely related to coronary spasm, for 3 reasons, such as (1) coronary flow normalized immediately after nitroglycerin infusion, along with visible dilatation of the epicardial vessel; (2) there was no decrease in LV function; and (3) troponin did not increase at 24 hours. These considerations exclude microembolization as the cause of the decrease in TIMI flow grade. No other adverse events occurred during cardiosphere infusion, whereas 1 pig died of severe hypotension followed by cardiac arrest immediately after placebo infusion. Analysis of the MRI performed in that pig before infusion revealed a major myocardial scar involving both ventricles with severe biventricular dysfunction. Therefore, only 14 pigs were analyzed at the 1 month post infusion end point.

Preclinical Study: Benefits Associated With Cardiosphere Infusion

Having established a safe dose, we performed a preclinical study, in which animals were blindly allocated to cardiosphere (50×10⁶ SCE, 1.3 cardiospheres; n=7) or vehicle infusion (n=8). The average diameter of the cardiospheres infused was 45±23 μm; 30.2±6.8%, 3.4±1.8%, and 0.24±0.17% of the cardiospheres were >50, 100, and 150 μm, respectively. Viability of the infused cardiospheres was 92.1±3.9%. MRI characteristics of the animals before treatment are shown in Table I in the Data Supplement.

LV Function Preservation and Scar Reduction

Figure 4A shows representative nonenhanced MR images at end-diastole and end-systole in placebo (left) and cardiosphere groups (right). Pooled data (Figure 4B) reveal that, at baseline, LVEF was similar in the 2 groups. At 1-month follow-up, however, LVEF was preserved in cardiosphere-treated animals (P=0.35 within group), whereas it significantly decreased in placebo (P=0.02 within group; P=0.004 between groups). In parallel, both indexed LV end-systolic and end-diastolic volumes significantly increased in the placebo group but not in the cardiosphere group (Figure 4C), indicative of attenuated adverse remodeling with cardiosphere treatment. In accordance with preservation of LV volumes, cardiosphere-treated pigs showed better 1-month regional function (assessed by end-systolic thickening) compared with placebo both in infarcted and in remote zones (Figure 4D and 4E).
Analysis of late gadolinium-enhanced images (Figure 5A) revealed that scar mass decreased significantly in cardiosphere-treated animals ($P=0.02$ within group) but not in placebo ($P=0.18$ within group; $P=0.002$ between group) leading to a 5-fold higher relative decrease in the cardiosphere group ($\approx11.6\pm2.7\%$ versus $\approx2.3\pm4.6\%; P=0.003$), despite similar scar mass at baseline (Figure 5B and 5C). Consistently, the decrease in scar size (scar mass divided by total LV mass; Figure 5D) was significantly greater in cardiosphere group than in control. In addition to the reduction of scar mass, we observed a strong trend toward a higher increase in viable mass in cardiosphere-treated animals (Figure 5B and 5D). We have previously noted that viable mass recovery tends to lag behind the reduction of scar, such that changes in viable mass that are not significant at 1 month (as is the case here) tend to increase with longer follow-up.\textsuperscript{11,13,14}

**Myocardial Perfusion Increase**

Myocardial perfusion was assessed using 2 different methods: (1) coronary flow reserve of the infarct-associated artery before cardiosphere infusion and 1 month later using a Doppler wire and (2) first-pass MRI to quantify perfusion in the infarcted area. Coronary flow reserve values at end point were higher in cardiosphere-treated pigs than in control, despite similar baseline values (Figure 6A). Consistent with these findings, MRI first-pass sequences revealed a significant increase in myocardial perfusion at end point in the cardiosphere group but not in placebo controls (Figure 6B; $P=0.02$ in cardiosphere versus $P=0.18$ in control, $P=0.13$ for intergroup comparison).

**Histology: Fibrosis, Vascular Density, and Architecture**

Functional measurements reviewed above indicate an increase in myocardial perfusion with cardiosphere treatment. Consistent with these findings, histological analysis revealed 2-fold higher arteriolar density (ie, isolectin and $\alpha$-smooth muscle actin-positive vascular structures) in the infarcted and border zones of cardiosphere-treated pigs compared with controls, but no differences in the remote zone (Figure 6C and 6D). Capillary density was also higher in the peri-infarct zone but not in the remote zone (Figure 6E).

MRI also revealed a decrease in myocardial scar after cardiosphere infusion. To confirm this histologically, we first evaluated the transmurality of the scar using Masson trichrome staining. Then, collagen content in the infarcted, border, and remote areas was quantified using Picrosirius red (which offers superior contrast between fibrotic and nonfibrotic areas in the remote and border zones relative to Masson trichrome, as evident from the representative images in Figure 7A1–7A4). Scar transmurality was less in cardiosphere-treated pigs versus placebo (Figure 7B). In parallel, collagen content was reduced in the cardiosphere-treated pigs not only in the infarct zone and in border zone but also in the remote zone (Figure 7C–7E).

These differences in fibrosis and vascularity were not accompanied by cardiomyocyte hypertrophy, as demonstrated by the similar cross-sectional areas of cardiomyocytes from cardiosphere and placebo pigs (Figure III in the Data Supplement).

**Hemodynamic Improvement**

To investigate whether the observed functional and architectural improvements were associated with hemodynamic improvement, we measured LVEDP and cardiac output (CO, from stroke volume and heart rate). At baseline, LVEDP and CO were similar in cardiosphere- and placebo-treated pigs. After treatment, LVEDP significantly decreased in cardiosphere but not in placebo, whereas CO increased only in the cardiosphere group (Figure 8A; Table). We did not observe any differences in systolic or diastolic blood pressure or heart rate that might have confounded the LVEDP and CO measurements (Table).

**Immunologic Safety: Cellular Infiltration and Alloantibodies**

Histological analysis revealed more lymphocyte infiltration in the infarcted and border zone of the treated animals compared with
placebo (5/7 animals versus 0/7 animals; \(P=0.002\)). Importantly, no area of cardiomyocyte damage related to immune response was observed, indicating that no animal had a grade of immune reaction \(>1\) (Figure IV in the Data Supplement). Slightly higher levels of alloantibodies were observed in cardiosphere-treated animals as compared with placebo (Figure IV in the Data Supplement), but the significance of this observation is uncertain.

**Discussion**

Adverse remodeling is a major contributor to MI-related mortality and morbidity.\(^{15,16}\) Despite the improvement of care and faster reperfusion, mortality remains \(>10\%\) at 1 year.\(^{3}\) Here, we demonstrated the ability of 3D multicellular cardiospheres to halt the ongoing process of adverse remodeling in a clinically relevant porcine model of convalescent MI. Furthermore, we established the safety of the desirable intracoronary route for the administration of this cell product.

**Safety of Intracoronary Infusion**

Intracoronary infusion is an easy, convenient, and widely available technique for delivery of biological products. Before this study, intracoronary infusion of cardiospheres had been avoided because of the prevailing assumption that these multicellular microtissues may cause microvascular obstruction resulting in myocardial damage. Indeed, some authors have raised the concern that intracoronary infusion of particles \(>10\ \mu m\) may be unsafe because of microvascular obstruction.\(^{17}\) However, the safety of intracoronary infusion of large particles (CellBeads of 160–400 \(\mu m\)) has previously been reported, although the number of particles infused was low (\(\leq 60000\)), and those studies aimed to cause a certain degree of microvascular occlusion.\(^{18–20}\) Here, we have been able to deliver intracoronary \(>1\times10^6\) cardiospheres with an average diameter of 45 \(\mu m\) without any adverse events. The first study evaluating intracoronary infusion of dispersed CDCs used minipigs with convalescent MI and traditional stop-flow delivery; intracoronary infusion of \(\geq25\times10^6\) CDCs, which have a diameter \(\approx 20\ \mu m\), led to elevation of serum troponin I at 24 hours, establishing the maximal safe dose as \(12.5\times10^6\) (or \(\approx 300000\) CDCs/kg).\(^8\) The combination of careful cardiosphere formulation and the use of continuous-flow infusion\(^2\) allowed us to deliver up to an equivalent of \(50\times10^6\)

![Figure 6](image-url)
cells intracoronary in the same model. This ability to deliver a higher number of cells may augment efficacy, although cell therapy does not necessarily follow conventional dose–response relationships.22–24

Antifibrotic and Proangiogenic Properties of Cardiospheres

CDCs have been extensively studied preclinically, leading to several phase I/II clinical trials involving first autologous and, more recently, allogeneic products.9,25 Thus, the ability of CDCs to decrease scar mass and to increase viable tissue has been thoroughly validated. In the porcine post-MI model, we confirmed the impressive antiremodeling properties of cardiospheres that had been observed by intramyocardial injection in small-animal models.13,14,26–28 Indeed, cardiosphere infusion completely halted further LVEF deterioration. Moreover, we observed an improvement in regional function and a decrease in fibrosis both in infarcted and in noninfarcted zones, indicating a positive effect on adverse global remodeling, and not just a decrease in scar content of the infarcted area. These global effects on LV remodeling and fibrosis may be related to the higher engraftment and survival of cardiospheres as compared with CDCs and to the secretion of growth factors, endoglin and MMP described.12,13,27,29,30

Taken together, these properties may enable a more sustained and diffuse paracrine effect. Paracrine signaling is a major contributor to cardiac regeneration after cell therapy.29,31–35 As benefits far outlast measurable cell engraftment,7,10,28 it seems likely that the benefit observed in this study might increase over time8,10 and potentially surpass the benefit observed with CDCs.11 The benefits seen here are indeed generally superior to those seen 1 month after intracoronary infusion of CDCs or after intramyocardial injection of a comparable dose of cardiospheres (comparison with historical results from Yee et al,9 and unpublished 1-month follow-up results from the study by Malliaras et al11 are summarized in Figure 8B and 8C). However, a direct comparison was beyond the scope of this study.

In addition to this antifibrotic effect, we describe a strong angiogenic effect after cardiosphere infusion. Indeed, we observed a 2-fold greater number of arterioles in the infarcted and border zones of cardiosphere-treated animals compared with placebo. This increased arteriolar number was associated with improved perfusion in the infarcted area as assessed by 2 different methods (invasive and noninvasive). Given the chronicity of the model, the increase in myocardial perfusion is probably attributable to the growth of new vessels, not to preservation of existing vasculature. This impressive angiogenic effect might be useful to recruit in selected clinical situations, for example, in patients with intractable angina or microcirculatory deficits.

Hemodynamic Improvement

Consistent with the structural changes, we observed hemodynamic improvements after cardiosphere infusion, including increased CO and, importantly, decreased LVEDP. High LVEDP is a strong predictor of adverse events (ie, acute heart failure or death)36–39; if reproduced in patients, the 2-fold decrease observed in this study could be highly meaningful clinically.
The structural and functional improvements associated with allogeneic cardiosphere infusion were associated with minimal immune reaction, confirming the safety of allogeneic heart–derived cell therapy. Although no immunosuppressive treatment was given, we did not observe any reaction >grade 1 of the International Society for Heart & Lung Transplantation (ISHLT) in the tissue, and the level of alloantibodies that were detected was not different than in the negative control.

Limitations
This study has several limitations. First, the MI model, although mimicking human adverse remodeling, is made on a background of young healthy animals. Therefore, their ability to recover and generate new cardiac tissue might be superior to what can be observed in patients with cardiovascular disease risk factors and other comorbidities. Second, engraftment was not quantified in this study; engraftment of cardiospheres has been shown to be greater than that of CDCs, but only in intramyocardial injection models. However, it is not clear whether the benefit of cardiospheres is related to the higher engraftment, to properties of the secretome, or both. Although engraftment correlates roughly with efficacy, the relationship is vague, particularly over the longer term. Nevertheless, increased short-term engraftment would be expected to boost the availability of secreted factors, so the 2 possibilities are undoubtedly intertwined.

Conclusions
We have demonstrated the safety of intracoronary infusion of multicellular 3D cardiospheres. Moreover, cardiosphere treatment decreases scar, attenuates adverse remodeling, increases myocardial perfusion, and improves hemodynamics in a pig model of convalescent MI. Cardiosphere infusion may deserve testing as a therapeutic approach to attenuate adverse remodeling or to achieve angiogenesis in humans.

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Disclosures
Drs E. Marbán and L. Marbán own equity in Capricor Inc. Drs L. Marbán, Kreke, and Smith are employed by Capricor Inc. The other authors report no conflicts.

Table. Baseline and End Point Values of LVEDP, CO, SBP, DBP, and HR in CSp- and Placebo-Treated Pigs (n=7 Per Group)

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<th>Baseline</th>
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<th>End Point</th>
<th>CSp</th>
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<th>CSp</th>
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<td>LVEDP, mm Hg</td>
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<td>CO, L/min</td>
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<td>SBP, mm Hg</td>
<td>71±11</td>
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<td>0.61</td>
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<td>18±16</td>
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<td>DBP, mm Hg</td>
<td>51±5</td>
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<td>63±5</td>
<td>67±16</td>
<td>0.36</td>
<td>13±3</td>
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<td>HR, bpm</td>
<td>108±11</td>
<td>111±27</td>
<td>0.84</td>
<td>104±16</td>
<td>98±7</td>
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<td>-6±18</td>
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CO indicates cardiac output; CSp, cardiospheres; DBP, diastolic blood pressure; HR, heart rate; LVEDP, left ventricle end-diastolic pressure; and SBP, systolic blood pressure.


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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Anesthesia

For all procedures, animals were anesthetized with acepromazine (0.25mg/kg), ketamine (20mg/kg) and propofol (0.83mg/kg) for the induction, followed by 1-3% isoflurane. Analgesia was achieved with oral carprofen (4mg/kg) and intra-muscular injection of buprenorphine (0.1mg/kg).

MRI

Myocardial scar mass, scar size (scar mass divided by LV mass), viable mass (total LV mass minus scar mass), LV volumes, LVEF, regional function and regional perfusion were measured using validated image processing software (Cvi42, Circle Cardiovascular Imaging Inc., Calgary, Canada) by a researcher blinded to treatment allocation. All volumes were indexed to body surface area (BSA) using the formula BSA=0.121*weight^0.575. Six-millimeter short-axis slices were acquired from the apex to the mitral valve plane. LV volumes, global and regional function were assessed using ECG-gated, breath-hold, cine steady-state free precession acquisitions. Stroke volume (SV) was derived from LV volumes and used to calculate cardiac output (CO=SV*HR where HR is heart rate). Scar mass and scar size were calculated using delayed contrast-enhanced sequences (acquired 8min following IV injection of Gd-based contrast agent). The scar area was defined using the full width at half maximum criterion by including all pixels with > 50% maximal signal intensity. Myocardial regional perfusion was assessed using first-
pass imaging immediately following IV injection of Gd, with 3 mid-LV short-axis slices acquired every 2-4 beats. For measurement, the average upslope to peak intensity was normalized to the LV blood pool intensity. Each short axis slice was divided into 6 segments, and the segments of infarcted area were determined by comparing each slice to the corresponding late-enhancement slice.

Regional function was analyzed by end-systolic thickening quantification. Regional segmentation was made using the AHA segmentation model\(^1\); the infarcted area was defined as segments vascularized by the LAD after the first diagonal branch (infarcted area), i.e. apical anterior, apical septal, mid antero-septal and mid anterior segments.

**Coronary flow reserve (CFR) measurement**

Intracoronary flow velocity was measured using Flowwire® (Volcano Corporation, San Diego, CA, USA) as described\(^2,3\) prior to cell infusion and before euthanasia. Nitroglycerine (0.2mg) was injected to prevent vasospasm. The Doppler flow wire was placed in the mid-LAD after the first diagonal branch. Velocity signal was recorded at rest and at peak hyperemia (induced by intra-coronary bolus of 60 µg adenosine). CFR was calculated as the ratio of highest average peak flow velocity over baseline average peak flow velocity (mean of 3 successive heart beats). At least 2 representative measurements were performed at each time point.

**Histology**

Histological analyses were performed on 8 µm sections from myocardial samples (fixed in 10% formalin and embedded in paraffin) obtained from infarct and border zone (3-6 slides per heart) and remote zone (2 slides per heart). The border zone was defined as the region at the edge of the
scar (comprising both viable and scar areas). Morphometric analysis and fibrosis were quantified with Masson’s trichrome staining and Picosirius red staining (8 slides/heart for each). Immune reaction in the heart was investigated using hematoxylin and eosin staining; cellular infiltration was graded using the International Society for Heart and Lung Transplantation (ISHLT) grading system by an experienced cardiac pathologist blinded to treatment allocation (DL)12. For vascular density quantification, myocardial samples underwent immunostaining for Isolectin B4 (Invitrogen I21411) and α-smooth muscle actin (5-10 images/slides). Arteries and arterioles were identified by Isolectin and α-smooth muscle actin-positive staining and capillaries by Isolectin positive staining only. All arterioles with diameter<300µm were counted. Arteriole and capillary densities were quantified in the infarcted, border and remote zones, and capillary density was quantified in the peri-infarct zone (i.e. viable zone of the infarcted and border zones) and in the remote zone. For cardiomyocyte cross-sectional area, we immunostained with wheat-germ agglutinin (Invitrogen) and α-sarcomeric actinin (ab9465 Abcam; 5-10 images/slide). Cross-sectional area was measured only in regions where myocytes met the following 3 criteria: cellular cross-section present; visible nuclei located in the center of the cell; and intact cell borders. Alexa Fluor-conjugated secondary antibodies were used and all slides were counterstained for DAPI (both Molecular Probes). All slides were imaged using a confocal laser microscope and analyzed using Image J software.

**Circulating anti-donor antibodies**

To assess humoral immune response, serum samples were collected from the recipient animals 4 weeks post infusion and screened for circulating anti-donor IgG using flow cytometry. Serum from an animal injected subcutaneously with 190 million PBMNCs (collected 2 weeks after injection) was used as a positive control.
SUPPLEMENTAL TABLE

Supplemental Table 1: MRI characteristics of the pigs before treatment (3-4 weeks after MI).

LVEDV: Left ventricle end diastolic volume; LVESV: Left ventricle end systolic volume;
LVEF: Left ventricle ejection fraction.

<table>
<thead>
<tr>
<th></th>
<th>Placebo treated</th>
<th>Csp treated</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDV indexed (mL/m²)</td>
<td>75±9</td>
<td>79±11</td>
<td>0.28</td>
</tr>
<tr>
<td>LVESV indexed (mL/m²)</td>
<td>44±6</td>
<td>47±4</td>
<td>0.28</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>41.4±2.3</td>
<td>40.2±2.6</td>
<td>0.28</td>
</tr>
<tr>
<td>Scar mass (g)</td>
<td>11.0±2.6</td>
<td>11.5±2.6</td>
<td>0.75</td>
</tr>
<tr>
<td>Scar size (%)</td>
<td>17.0±2.3</td>
<td>18.1±2.5</td>
<td>0.41</td>
</tr>
<tr>
<td>Viable mass (g)</td>
<td>54.4±7.1</td>
<td>51.9±9.3</td>
<td>0.65</td>
</tr>
</tbody>
</table>
Supplemental figure 1: (A) number and size of secondary CSp manufactured from 12.5M CDCs
Assessment of cardiosphere loss during catheter infusion. (B) design of the study; (C) number of CSP before and after infusion through the catheter and (D) % loss during the infusion.
Supplemental figure 2: Representative pictures of TTC stained heart slices 24 hours after infusion of 50M single cell equivalent in a naïve pig
Supplemental figure 3: (A) Images of cardiomyocytes for calculation of cross-sectional area in CSp- and placebo-treated pigs. No differences were observed between CSp and placebo in the infarcted (B), border (C) and remote (D) zones. (N=7 per groups)
Supplemental figure 4: Immunological safety. (A) Cellular infiltration in the infarcted, border and remote areas; CSp infusion causes a slight immune reaction in the tissues but without any myocyte damage (ISHLT grade<2). (B) Allo-antibody quantification by flow cytometry; we observed a small elevation of allo-antibodies in CSp treated pigs compared to the negative control (placebo-treated pigs, light blue curve); however these values are much lower than those in the positive control (red curve).
SUPPLEMENTAL REFERENCES

