Cellular Postconditioning
Allogeneic Cardiosphere-Derived Cells Reduce Infarct Size and Attenuate Microvascular Obstruction When Administered After Reperfusion in Pigs With Acute Myocardial Infarction

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Background—Intracoronary delivery of cardiosphere-derived cells (CDCs) has been demonstrated to be safe and effective in porcine and human chronic myocardial infarction. However, intracoronary delivery of CDCs after reperfusion in acute myocardial infarction has never been assessed in a clinically-relevant large animal model. We tested CDCs as adjunctive therapy to reperfusion in a porcine model of myocardial infarction.

Methods and Results—First, escalating doses (5, 7.5, and 10 million cells) of allogeneic CDCs were administered intracoronary 30 minutes after reperfusion. Forty-eight hours later, left ventriculography was performed and animals euthanized to measure area at risk, infarct size (IS), and microvascular obstruction. Second, identical end points were measured in a pivotal study of minipigs (n=14) that received 8.5 to 9 million allogeneic CDCs, placebo solution, or sham. Multiple indicators of cardioprotection were observed with 7.5 and 10 million allogeneic CDCs, but not 5 million CDCs, relative to control. In the pivotal study, IS, microvascular obstruction, cardiomyocyte apoptosis, and adverse left ventricular remodeling were all smaller in the CDC group than in sham or placebo groups. In addition, serum troponin I level at 24 hours was lower after CDC infusion than that in the placebo or sham groups, consistent with the histologically-demonstrated reduction in IS.

Conclusions—Intracoronary delivery of allogeneic CDCs is safe, feasible, and effective in cardioprotection, reducing IS, preventing microvascular obstruction, and attenuating adverse acute remodeling. This novel cardioprotective effect, which we call cellular postconditioning, differs from previous strategies to reduce IS in that it works even when initiated with significant delay after reflow. (Circ Heart Fail. 2015;8:322-332. DOI: 10.1161/CIRCHEARTFAILURE.114.001484.)

Key Words: acute myocardial infarction ■ animal models ■ coronary interventions ■ ischemic postconditioning

Percutaneous coronary intervention has become standard therapy worldwide for patients with acute myocardial infarction (AMI), not only reducing short-term adverse cardiac events but also improving long-term clinical outcome.1 Nevertheless, many patients with AMI progress to heart failure (HF) even with best current therapy. Adverse left ventricular (LV) remodeling after AMI is a precursor to the development of overt HF and is an important predictor of mortality.2,3 Prominent among the factors that underlie adverse remodeling is microvascular obstruction (MVO) after reperfusion.4,5 a state of myocardial tissue hypoperfusion despite patent epicardial coronary arteries. Severe MVO may lead to no-reflow, which is an independent predictor of adverse clinical outcome after AMI.5-7 Numerous strategies have been tested to reduce infarct size (IS), but, once reflow has occurred, nothing seems to work. All successful pharmacological strategies must be applied prior to reperfusion.8 Ischemic postconditioning (created by cyclic intracoronary balloon inflations) requires immediate manipulation of flow at the time of reperfusion, with loss of benefit if there is any significant delay.9,10

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Cell therapy has the potential to revise the paradigm, but little is known about the utility and risks of intracoronary cell administration soon after (ie, within 30 minutes of) reperfusion. No clinical data are available; most cell therapy clinical trials have infused cells 1 to 14 days post-AMI. By that time, cardiomyocytes at risk are already dead, so that there is limited potential (if any) for myocardial salvage. Given the delays intrinsic to autologous tissue harvesting and cell processing, applications in the acute reperfusion phase will require allogeneic (off-the-shelf donor-derived) products. Preclinical studies of acutely administered allogeneic mesenchymal stem cells (MSCs) or their precursors have yielded variable results. Although heart-derived stem cells have been tested in both large animals and humans in chronic ischemic settings, the only studies in an acute ischemia/reperfusion model were in rats, where structural and functional outcomes were improved dramatically by the intracoronary infusion of cardiosphere-derived cells (CDCs) 20 minutes post-AMI. However, the 3-week end point in those studies made it impossible to separate cardioprotection from regeneration.

We sought to determine whether early infusion of allogeneic CDCs during reperfusion leads to myocardial protection in a clinically-relevant large animal model. To avoid possible ischemic postconditioning due to balloon inflations, and to mimic clinical reality, the initiation of therapy was delayed until 30 minutes post-reperfusion. We used a 48-hour end point to assess acute cardioprotection without the confounding effects of tissue regeneration, which may contribute to the final outcome of longer-term studies.

Figure 1. Study protocols. Dose-escalation study (A) and pivotal study (B). AMI indicates acute myocardial infarction; CDCs, cardiosphere-derived cells; LVG, left ventriculography; and TnI, troponin I.
Figure 2. Comparison of different doses of cardiosphere-derived cells (CDCs) for assessment of functional and histological efficacy. Representative left ventriculograms at each dose of CDCs (A). Arrow indicates preserved wall motion in the border zone 48 hours postintervention. Yellow lines indicate a contour of end-diastolic and end-systolic left ventricular (LV) endocardial margins. Pooled change in left ventricular ejection fraction (LVEF; B), left ventricular end-diastolic volume index (LVEDVI; C), and left ventricular end-systolic volume index (LVESVI; D) between baseline and 2 days post CDCs infusion with escalating dosage. Comparison of area at risk (AAR; E), infarct size (IS; F), and microvascular obstruction (MVO; G, as % of AAR) 48 hours postinfusion. P value is the result of Kruskal–Wallis test. *P<0.05 and **P<0.01 between 2 groups.
Methods
For detailed methods, please see the Data Supplement. All animal studies were performed with approval from the Institutional Animal Care and Use Committee of the Cedars-Sinai Health System. Three separate experimental protocols were performed, as depicted schematically in Figure 1 and in Figure IA in the Data Supplement. A total of 15 Yucatan minipigs were studied in a dose-escalation study (Figure 1A); 14 completed the pivotal study (Figure 1B); and 6 pigs were dedicated to the measurement of cell engraftment (Figure IB in the Data Supplement). One Sinclair minipig (#0111) served as the donor for allogeneic heart-derived cells.

Cell Culture
Allogeneic CDCs were grown from a freshly-explanted heart obtained from 1 male Sinclair minipig.

Myocardial Infarct Creation and CDC Infusion
AMIs were created in adult Yucatan minipigs by inflation of an angioplasty balloon (TREK OTW 2–3 mm, Abbott Vascular, Santa Clara, CA) in the mid-left anterior descending artery (distal to the first diagonal branch) for 1.5 hours. Two studies were performed contemporaneously, involving a total of 39 minipigs: a dose-escalation study and a pivotal study. Because of acute reperfusion-related mortality (n=4) or technical reasons detailed in the Results section (n=6), 10 animals failed to complete the protocol.

Fifteen minipigs completed the dose-escalation study. Allogeneic CDCs (5, 7.5, and 10 million cells; n=5 pigs per dose) were formulated, frozen, and count-verified just prior to intracoronary infusion, which was performed 30 minutes post-reperfusion via an over-the-wire balloon catheter, placed in the mid-left anterior descending artery. From the dose-escalation cell counts, post-thaw cell recoveries were 69.6±1.5% and cell viability 90.7±0.4% (n=7). The CDCs were administered in 3 equally divided cycles of balloon inflation separated by 3 minutes of deflation (Figure 1A).¹⁶

Fourteen minipigs completed the pivotal study and were randomized to receive CDCs (n=4, with the cell infusion procedure as in the dose-escalation study), placebo (n=5), or sham (n=5; balloon placement in the left anterior descending artery but without inflations, to exclude possible confounding effects related to ischemic postconditioning). The latter 2 groups, which had similar distributions and medians, were statistically...
Figure 4. Allogeneic cardiosphere-derived cells (CDCs) attenuate infarct and no-reflow size. Representative transverse cardiac slices stained with Gentian violet and 2,3,5-triphenyl tetrazolium chloride (A). Area at risk (AAR; as % of left ventricular [LV]; B), infarct size (IS; as % of AAR; C), and IS (as % of LV; D) at 48 hours postintervention. Representative transverse cardiac slices stained with Thioflavin-T under ultraviolet light (E). No-reflow area (as % of AAR) 48 hours postintervention (F). *P<0.05 between 2 groups. MVO indicates microvascular obstruction.
indistinguishable (P=0.11–0.59 in all of histological and functional indicators) and thus were pooled as controls for the dosing study. The pivotal study was designed to mimic a clinical situation in which preformulated bags of CDCs containing a nominal dose of 12.5 million CDCs were frozen and thawed for administration, without cell counting prior to infusion; based on the post-thaw cell recovery data in the dose-escalation study, actual delivered total cell dosage was estimated at ≈8.7 millions.

**Left Ventriculography**
To measure global LV function and volumes, left ventriculography was performed before infusion and prior to euthanasia on day 2 using a pigtail catheter inserted retrogradely into the LV, with imaging in the 40° left anterior oblique projection (30 frames per second) using nonionic contrast (27–30 mL/3 s). LV volumes were indexed to body surface area, calculated as per the formula (body surface area=0.121 × body weight kg^0.575).21

**Safety Evaluation (Coronary Flow and Arrhythmia)**
Coronary flow was evaluated using thrombolysis in myocardial infarction (TIMI) score and corrected TIMI frame count grading systems. ECGs were recorded before induction of AMI, before CDC/vehicle infusion, immediately after CDC/vehicle infusion, 1 hour after CDC/vehicle infusion (only in pivotal study), and prior to euthanasia to monitor for arrhythmias. In addition, ECGs were continuously measured during intracoronary infusion and 1 hour postintervention for arrhythmias.

**Histopathologic Evaluation**
Two days after CDC or vehicle infusion, minipigs underwent median sternotomy and direct injection of dyes into the left atrium (Gentian Violet and Thioflavin T) to assess area at risk (AAR) and MVO. Then, coronary flow was evaluated using thrombolysis in myocardial infarction (TIMI) score and corrected TIMI frame count grading systems. ECGs were recorded before induction of AMI, before CDC/vehicle infusion, immediately after CDC/vehicle infusion, 1 hour after CDC/vehicle infusion (only in pivotal study), and prior to euthanasia to monitor for arrhythmias. In addition, ECGs were continuously monitored during intracoronary infusion and 1 hour postintervention for arrhythmias.

**Histopathological Evaluation**
Two days after CDC or vehicle infusion, minipigs underwent median sternotomy and direct injection of dyes into the left atrium (Gentian Violet and Thioflavin T) to assess area at risk (AAR) and MVO. Then, minipigs were euthanized and the hearts excised and sectioned into 1-cm-thick short-axis slices to measure AAR, IS, and MVO. Each slice was imaged digitally; IS and MVO areas were determined by manual tracing by a researcher blinded to treatment allocation. The fraction of the AAR within the total area was equated with its weight percent of the total weight of LV. MVO and IS were expressed as the fraction of the AAR within the total area.

**Blood Examination**
In the pivotal study, blood draws were performed 24 hours after CDC or vehicle infusion and prior to euthanasia (48 hours) to measure serum troponin-I as a marker of cardiac injury.

**Engraftment Study**
Engraftment was measured (Figure IA in the Data Supplement) as described.22 Luciferase-labeled CDCs were delivered via intracoronary infusion (n=6) 15 minutes post-reperfusion. Animals were euthanized 15 minutes (n=3) or 48 hours (n=3) later and tissues were taken for luciferase measurement.

**Statistical Analysis**
Pooled data are expressed as box plots. Mann–Whitney U test was used for comparisons between 2 independent groups. Multiple groups were compared using Kruskal–Wallis test with Bonferroni post hoc testing. P<0.05 was considered statistically significant.

**Results**

**Adverse Events and Mortality**
In the dose-escalation study (Table I in the Data Supplement), 1 animal of 18 (6%) died of cardiogenic shock immediately after left ventriculography post-reperfusion. Two pigs were excluded due to technical failure or incomplete dye study. There was no further mortality (0%), nor were any adverse events noted during the infusion procedure in either group. In the pivotal study, 3 of 21 animals (14%) died during creation of AMI due to ventricular arrhythmia; 3 pigs were excluded due to technical failure of AMI creation or incomplete dye study. One pig was excluded due to incidentally-discovered hypertrophic cardiomyopathy at necropsy. Thus, a total of 14 pigs completed the pivotal study. There was no further mortality within the protocol (0%), nor were there any other adverse events (Table II in the Data Supplement).

**Dose-escalation Study**

**Functional and Histological Benefits of CDC Infusion With Optimal Dose**
To assess differences of treatment effects with escalating doses of allogeneic CDCs, we measured cardiac function and volumes by left ventriculography. Representative ventriculograms 48 hours post-CDC treatment show amelioration of LV anterior wall motion compared with control, especially in the 7.5 million pigs (Figure 2A, yellow arrows). Although the changes in the ejection fraction from baseline to 48 hours post-CDC infusion were not significantly different among groups (Figure 2B), minipigs in control and 5 million groups underwent progressive LV dilatation (both at end-diastole [Figure 2C] and end-systole [Figure 2D]); the change in left ventricular end-diastolic volume index was significantly attenuated in the pigs that received 7.5 million cells (Figure 2C). In addition, we evaluated AAR, IS, and MVO by dye study 48 hours post-infusion. Although AAR in the 4 groups was comparable, IS and MVO (as a % of AAR) were smaller (7.5 million) or strongly tended to be smaller (10 million) than in the control or 5 million groups (Figure 2E–2G).

**Pathological Assessment and Cytoprotective Effect of CDCs With Optimal Dosage**

The extent of intramyocardial hemorrhage was indistinguishable in all groups (Figure IIA and IIB in the Data Supplement). By contrast, CDC infusion blunted apoptosis as revealed by TUNEL staining (Figure 3A). The number of TUNEL-positive cardiomyocytes in the border zone was lower in all CDC groups than in control ($P=0.002$; Figure 3B, left panel), a pattern repeated, albeit with less significance ($P=0.052$), in the ischemic zone (Figure 3B, right panel).

As for coronary flow evaluation, absolute corrected TIMI frame counts post-infusion were different in the 4 groups ($P<0.05$; Table I in the Data Supplement), with significant pairwise differences between control and 10 million groups, but no differences among control, 5 million, or 7.5 million groups (Figure III in the Data Supplement).

Overall, although there is a tendency for the 10 million group to be worse than the 7.5 million group, both 7.5 and 10 million groups seem to be better than control or 5 million group in terms of functional, histological, and safety concern. We conclude that 7.5 to 10 millions is the optimal therapeutic dosage range.

**Pivotal Study**

Having established a target dosage range of 7.5 to 10 million CDCs, we performed a pivotal preclinical study with animals randomized to placebo, sham, or CDC groups. The CDC group mimicked the clinical situation in which a preformulated bag is acutely thawed for infusion at the site of reperfusion. It is clinically unrealistic to perform cell counting prior to product release in the AMI setting, so that infusion bags filled with 12.5 million CDCs were used, without on-site cell counting.

![Figure 6. Allogeneic cardiosphere-derived cells (CDCs) decreased apoptosis of cardiomyocytes assessed by TUNEL staining. Comparison of apoptosis of cardiomyocytes assessed by TUNEL staining (A) and quantitative evaluation of TUNEL-positive cell number in border and infarct zones (B). $P$ value is the result of Kruskal–Wallis test. *$P<0.05$ between 2 groups. DAPI indicates nuclei. DAPI, 4',6-Diamidino-2-Phenylindole, Dihydrochloride; SA, α-sarcomeric actinin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; and WGA, wheat-germ agglutinin.](http://circheartfailure.ahajournals.org/Downloaded from)
counting and recognizing that some of the cell product would be retained in the dead space of the bags and tubing. From the cell count findings in the dose-escalation study, actual delivered cell dosage was estimated at ≈8.7 millions, well within the desired therapeutic range of 7.5 to 10 millions.

**Histological Efficacy of Intracoronary Infusion of CDCs as Adjunctive Therapy to Reperfusion**

To assess the acute benefit of allogeneic CDCs, we evaluated histological efficacy of CDCs by dye study at 48 hours. Figure 4A shows representative slices stained with gentian violet to delineate AAR and 2,3,5-triphenyl tetrazolium chloride to quantify IS.23 The infarcted zone (white/yellow) within the AAR (brick-red) is visibly smaller than that of sham or placebo. The AAR/LV (as a % of the LV) was similar among all 3 groups, indicating comparable degrees of initial injury (Figure 4B). IS (as a % of AAR) was smaller in the CDC group (59.7%, P<0.05 versus sham and placebo) compared with sham (81.0%) or placebo (80.3%; Figure 4C). In addition, IS (as a % of the LV) was also smaller in the CDC group (12.5%, P<0.05 versus sham and placebo) than in sham (19.9%) or placebo (18.5%) groups (Figure 4D).

We also evaluated MVO in the pivotal study. Figure 4E shows ultraviolet epifluorescent images of representative slices stained with thioflavin T; here, the MVO area appears dark as it has not taken up the infused thioflavin and thus is nonfluorescent. MVO area is distinctly smaller in the CDC-treated pig than in sham or placebo, as verified by the pooled data in Figure 4F.

**Functional Efficacy in the Acute Phase of Intracoronary Infusion of Allogeneic CDCs**

We assessed the acute treatment effects of allogeneic CDCs on LV function and volumes by left ventriculography. Although the change of ejection fraction (Figure 5A) between baseline and 48 hours postintervention was similar in all groups, minipigs in sham and placebo groups underwent progressive LV dilatation (Figure 5B and 5C), which was attenuated in CDC-treated pigs.

**Safety Evaluation of Intracoronary Infusion of CDCs**

Consistent with the structural evidence of reduced myocardial injury (Figure 4), CDC treatment decreased serum troponin I level measured 24 hours post-MI (Figure 5D). These data support the conclusion that CDCs exert a cardioprotective effect, even when cell administration is delayed to 30 minutes after reperfusion.

**Pathological Assessment and Cytoprotective Effect in Pivotal Study**

The extent of intramyocardial hemorrhage was comparable in the 3 experimental groups (Figure IVA and IVB in the Data Supplement). We also quantified apoptosis in the 3 groups by staining border and infarct zone samples for TUNEL (Figure 6A). Consistent with the results of the dose-escalation study, the percentages of TUNEL-positive myocytes in the border (49.5%) was lower in CDC-treated hearts than in sham (68.8%) or placebo (64.3%; Figure 6B, upper panel). Here, we additionally see unequivocal benefit in the infarct zone (Figure 6B, lower panel).

**Measurement of CDC Engraftment by Luciferase**

Cardiac engraftment of CDCs decreased from 39.9±9.6% (n=3) at 15 minutes to 3.7±1.6% (n=3) at 48 hours. There was a significant difference in engraftment at the 2 time points (Figure IB in the Data Supplement).

**Assessment of Vascularization**

We analyzed vessel density in border zone samples by immunostaining in dose-escalation and pivotal studies (Figure VA and VB in the Data Supplement). Quantification of vessel density did not reveal any significant differences among groups.

**Discussion**

Early and successful myocardial reperfusion with percutaneous coronary intervention is the most effective strategy for AMI.24,25 However, reperfusion itself has the potential to aggravate injury, which may in part explain why the incidence of HF after AMI approaches 25%.26 Here, we have demonstrated a novel benefit of infused CDCs administered 30 minutes after reperfusion: such treatment decreased IS and MVO in pigs with AMI. Our findings are notable for 2 reasons: first, CDCs work despite having been administered relatively late after reperfusion. No other cardioprotective modality has successfully reduced IS without pretreatment (ischemic or pharmacological preconditioning) or immediate intervention on reopening the affected artery (ischemic postconditioning). Second, our study design, with 48-hour structural and functional end points, guarantees that we are studying acute cardioprotection; otherwise, it is impossible to exclude a partial or dominant contribution from longer-term regenerative effects of the cells, which are evident only weeks after treatment.20 Given the novelty of the phenomenon described here, we call it cellular postconditioning.

**Influence of Postconditioning on Reperfusion Injury**

Ischemic postconditioning, described by Zhao et al,27 is a cardioprotective phenomenon which can be recruited by applying intermittent cycles of ischemia immediately after reperfusion. There is some inconsistency in the literature as to how long a delay after reperfusion can be imposed while retaining the benefits of ischemic postconditioning, with most studies claiming rapid evanescence (<10 minutes).9,10 but one showing that delays of ≤30 minutes may be possible.28 To see whether ischemic postconditioning might have influenced our own results, we compared the placebo group (where 3 cycles of stop-flow ischemia were applied without cell infusion) with the sham group, which simply had a catheter placed in the left anterior descending artery without intermittent balloon inflations. Results in the 2 groups were indistinguishable, ruling out a contributory role for ischemic postconditioning in our protocol. Thus, the cardioprotective effects of CDCs infused at 30 minutes are distinct from those of ischemic postconditioning. Not only is the trigger different (cells versus ischemia) but also the window of treatment opportunity is longer. Such a delay is consistent
with clinical reality: when a patient presents with AMI, the immediate focus is on prompt recanalization of the occluded vessel. Only after patency has been re-established and a stent deployed will the typical clinician consider adjunctive therapy; the allowance of 30 minutes to initiate infusion accommodates decision to treat, product thawing, and technical preparation for administration.

Feasibility and Safety of Intracoronary Infusion of CDCs in AMI
In addition to the above considerations, translation will be facilitated by the fact that we used standard clinical equipment and intracoronary infusion for cell transplantation. Intracoronary infusion can be performed in a minimally invasive manner and has been safely used in numerous preclinical studies and clinical trials.17,18,22,29,30 Some previous large animal studies have questioned the safety of intracoronary infusion of stem cells post-MI, with decreased coronary flow and elevation of cardiac enzymes attributed to microvascular plugging.12-14,31 Houtgraaf et al15 had more favorable results after careful attention to cell dosage, size, and infusion rate. In terms of cell dosage, we have validated the safety of 12.5 million intracoronary CDCs in pigs with chronic ischemic cardiomyopathy, and 25 million CDCs administered to post-MI patients in the CARDiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction (CADUCEUS) trial, without complications such as microembolization.18,22 Cell dosage must be carefully validated in the acute phase of reperfusion post-MI, given the presence of infarct-related microvascular injury.32 As for cell size, although it is reported that the average diameter of MSCs is 30 to 50 μm,33 CDCs are ≈20 μm in diameter.22 In addition, previous studies demonstrated microinfarction or slow-flow phenomena at high infusion rates and high cell doses (eg, 50 million cells at a rate of 1.5 million cells/min).12,14 On the other hand, Houtgraaf et al15 found that slow infusion of mesenchymal progenitor cells (0.5 million cells/min) enabled intracoronary infusion of 50 million cells without compromise of coronary flow. Here, we infused relatively low cell numbers at rates of 0.8 to 1.6 million cells/min following reperfusion; 7.5 to 10 million CDCs at rates of 1.25 to 1.6 million cells/min were well-tolerated and therapeutically active, in general agreement with Houtgraaf et al.15 Our studies differ in that we delayed cell infusion until 30 minutes after reflow (Houtgraaf et al15 began infusion at 15 minutes of reflow) and in the timing of IS quantification: we focused on 48-hour end points, while Houtgraaf et al15 quantified IS only at 8 weeks, at which time longer-term regenerative effects may cloud the evaluation of cardioprotection.20

Potential Mechanism of Benefit in CDC-Treated Hearts
In the vast majority of experimental studies, the number of differentiated myocytes derived from transplanted stem cells is too small to account for the observed improvements in cardiac function.34 Thus, the prevailing concept of stem cell efficacy has shifted toward the paracrine hypothesis, according to which the transplanted cells are proposed to produce soluble factors that are beneficial to the infarcted heart.35 Indeed, skeletal myoblasts,36 bone marrow–derived cells,37 and cardiac-derived cells38 produce and secrete a broad variety of cytokines. In small comparative studies, CDCs outperformed MSCs in vitro39 and in vivo.40 In a head-to-head comparison of 4 different cell types (CDCs, bone marrow–derived mononuclear cells, bone marrow–derived MSCs, and adipose-derived MSCs) in the same animal model in the same laboratory, CDCs emerged as superior in terms of paracrine factor secretion, angiogenesis, cardiomyogenic differentiation, ischemic tissue preservation, antiremodeling effects, and functional benefit post-MI.41

Potential cardioprotective effects of paracrine factors include antiapoptotic effects on resident myocytes, upregulation of angiogenesis, modulation of inflammatory processes resulting in better infarct healing, improvements of cardiac metabolism and contractility, promotion of cardiomyocyte cell cycle re-entry, and induction of secondary humoral effects in the host tissue.42,43 Here, we observed histological benefits including reduction of IS, MVO, and apoptotic cardiomyocytes only 48 hours post–cell transplantation, which is consistent with paracrine effects but not differentiation of transplanted cells into cardiomyocytes or vessels, as demonstrated in Figures I and V in the Data Supplement. Beyond these considerations, however, the present study provides no insight into the detailed mechanisms of benefit of cellular postconditioning.

Limitations
This study has several limitations. First, this model is one of iatrogenic AMI caused by balloon occlusion. The pathophysiological situation differs from acute coronary syndrome with regard to influence of microembolization derived from atherosclerotic plaque or thrombus. Also, the well-defined duration of ischemia here differs from the clinical situation, which is much more heterogeneous. Second, we infused allogeneic CDCs in this study, but did not look for a potential immune response given the short-term nature of the end points. However, allogeneic heart-derived cells have been transplanted without eliciting deleterious immune reactions in previous reports.10,44,45 Although off-the-shelf allogeneic products are clearly necessary for the infusion of cells immediately after percutaneous coronary intervention, we need to test the safety concerns of allogeneic cells in longer-term experiments.

Conclusions
We demonstrate a cardioprotective effect of CDCs after reperfusion using a large animal model and strategies that are compatible with standard clinical practice. These results not only motivate potential clinical exploration of acutely-administered CDCs in AMI but also the further exploration of long-term efficacy and mechanism of benefit.

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References
Early reperfusion with percutaneous coronary intervention is the most effective strategy for treating acute myocardial infarction (MI). However, reperfusion itself has the potential to aggravate injury, which can progress to left ventricular remodeling and heart failure. Cell therapy clinical trials have focused on patients with MI in the subacute (days to weeks post-MI) or chronic (months) phases, at which time the opportunity for myocardial salvage has long passed. Here, we sought to determine whether early infusion of allogeneic cardiosphere-derived cells (CDCs) during reperfusion leads to myocardial protection in a clinically-relevant large animal MI model. CDCs mediate regeneration in chronic MI patients (CADUCEUS trial) and in various animal models; work in rodents also reveals a strong cardioprotective effect post-MI. Highly standardized off-the-shelf allogeneic CDCs have been used in 2 early-phase clinical trials of chronic heart disease (ALLSTAR and DYNAMIC) with no safety concerns to date. Intracoronary infusion of allogeneic CDCs 30 minutes after reperfusion decreased infarct size and suppressed microvascular occlusion, quantified by classical histological methods. Adverse remodeling, as assessed by left ventriculography, was also attenuated by post-MI CDC infusion. The effects were not due to ischemic preconditioning because an identical protocol of balloon inflations without CDCs did not mimic the benefits. No other adjunctive treatment has proven effective in limiting infarct size when delayed to 30 minutes post-reflow, a delay which is consistent with standard clinical practice. This novel cardioprotective effect of intracoronary CDCs, which we call cellular preconditioning, motivates clinical testing of a new treatment paradigm for acute MI in which allogeneic CDCs are administered adjunctively after reperfusion.
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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Culture of porcine CDCs

We obtained allogeneic CDCs from a Sinclair mini-pig (#0111) heart from Sinclair Bio Resources for the dose-escalation study, and the pivotal study to create a master cell bank (MCB). The heart arrived on ice submerged in cardioplegic solution. Hearts were grossly dissected and biopsy-sized pieces (~25mg) were seeded to create explant derived cells (EDCs). After ~14 days, the EDCs were harvested to create a MCB. MCB vials were thawed and cultured as cardiospheres (CSps) in suspension culture. CSps were grown on Ultra Low® Cell STACK® vessels (Corning Life Sciences). Allogeneic CDCs were grown by seeding CSps on Nunc Triple Flasks (Thermo Scientific), and passaging when confluent. Allogeneic CDCs were resuspended (1.25M/ mL for a total dose of 12.5M) in CryoStor™CS10 (BioLife Solutions) in cryobags (PL07 PermaLife Bags, OriGen Biomedical Inc), placed directly in a CryoMed controlled-rate freezer, and then transferred to liquid nitrogen. CDCs were thawed at the day of the infusion. Upon thawing, 1 mL of heparin (100 USP units/ mL) and 0.1 mL nitroglycerin (50 µg/ mL) were added as diluents for a total 10 mL dose for administration. Cell dose preparation was performed by Capricor, Inc.

MI creation and CDC infusion

On day 0, animals were premedicated with ketamine 20mg/ kg IM, atropine 0.05mg/ kg IM, and acepromazine 0.25mg/ kg IM. Animals were subsequently induced with propofol 2-4mg/
kg IV to effect, intubated, and maintained on isoflurane 2-3%. Amiodarone 10mg/ kg IV loading dose, then 0.5mg/ kg IV as needed and lidocaine 0.03μg/ kg/min were given for ventricular arrhythmias. Heparin 100IU/ kg IV was given for anticoagulation. Mini-pigs were subjected to an anteroseptal MI by inflation of an angioplasty balloon in the mid-left anterior descending artery (LAD) (distal to the 1st diagonal branch) for 1.5 h., followed by coronary reperfusion.

For the dose-escalation study, total 15 mini-pigs received escalating doses (exact cell number of 5, 7.5 and 10M cells) of allogeneic CDCs to find optimal dosage in terms of the safety and efficacy. For pivotal study, intracoronary infusion of allogeneic CDCs in thawed bags (estimated the cell number was approximately 8M due to cell death by freeze and thawing process) was performed thirty minutes post-MI using a standard clinical over-the-wire angioplasty balloon (TREK®, Abbott Vascular) in 3 boluses of 3.3 mL with intermediate washes of 2 mL between each bolus to ensure complete cell delivery.¹

**Dye infusion procedure and evaluation**

Two days after AMI and infusion of CDCs/ vehicle, pigs underwent induction of anesthesia and median sternotomy. An angioplasty balloon was placed at the treated lesion created during the MI procedure. With the balloon deflated, mini-pigs were infused with thioflavin T (50mls, 2% solution diluted with PBS) by direct injection into the left atrium over 60 seconds. After infusion of Thioflavin T was completed, the angioplasty balloon was inflated and mini-pigs were infused with Gentian violet (50mls, 1.6% solution diluted with 40mls PBS and 10mls ethanol) by direct injection into the left atrium over 60 seconds. Gentian violet stains the non-ischemic zone blue; the region of the heart that does that is not perfused by Gentian violet (i.e. does not stain blue)
represents the ischemic area (area at risk). Finally, mini-pigs were sacrificed and the heart was explanted and sectioned into 1 cm thick short-axis slices.

Thioflavin T is a fluorescent dye (visualized under UV light), which stains endothelium receiving blood flow. Areas of microvascular obstruction (areas of “no-reflow”) are not stained and appear as non-fluorescent area within the infarct zone. Then transverse ventricular slices were incubated with 2% TTC (2,3,5-triphenyl tetrazolium chloride) for 20 min at 37°C to stain viable myocardium. TTC stains viable myocardium brick red, while the infarct appears as non-TTC stained tissue (white/ yellow).

TIMI and cTIMI frame count grading system

Coronary flow of the LAD was evaluated pre and post cell infusion according to 4 grades of flow, as previously described. The corrected TIMI frame count (cTFC) is also an index of coronary blood flow that is easily obtained by counting the number of cine frames required for dye to travel from the ostium to a standardized distal landmark. The cTFC is a simple, reproducible, objective, and quantitative index of coronary flow that allows standardization of TIMI flow grades. In addition, it is reported that the cTFC is related to the early risk of adverse outcomes in acute coronary syndromes.

Ex vivo engraftment study

CDCs were transduced with an adenoviral vector carrying the firefly luciferase gene three days prior to injection. For each animal, a separate standard curve was constructed using the specific transduced cell preparation infused into that animal. All pigs were infused with 7.5
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million luciferase-labeled CDCs 30 minutes after reperfusion. Then, 15 minutes (n=3) or 48 hours (n=3) after cell delivery, the infused regions of the LV and RV were homogenized with 10% fetal bovine serum and analyzed for luminescence (Supplemental Figure 1A). Animal-specific standard curves were prepared by measuring the luminescence from known numbers of transduced cells, with conversion to a cell number by reference to the respective standard curve. Engraftment was expressed as a percentage of the number of cells originally infused (Supplemental Figure 1B).
## Dose-escalation study

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※cTFC; corrected TIMI frame count
Supplementary Table 2.

**Pivotal study**

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※cTFC; corrected TIMI frame count
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Supplementary Figure 1.

A

Luciferase-labeled CDCs infusion (i.c.)

AMI

90 min. occlusion 30 min. 15 min.

Reperfusion

Sacrifice 15min.

Luciferase assay (n=3)

Sacrifice 48hrs

B

Engraftment

(\%)

* 50

40

30

20

10

0

15min. (n=3) 48hrs (n=3)
Supplementary Figure 2.

(A) Representative images of the border zone and infarct zone under different conditions. Scale bar = 100 μm.

(B) Graph showing the area under the curve (AU) for the border zone and infarct zone across different conditions. * indicates significant difference. P-values: p = 0.039 for the border zone, p = 0.61 for the infarct zone.
Supplementary Figure 3.

The diagram shows the cTIMI frame count with error bars. The x-axis represents different conditions: Control, 5M, 7.5M, and 10M. The y-axis represents the frame count. The control group has a lower frame count compared to the other groups. The p-value for the comparison is 0.035.
Supplementary Figure 4.

A

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B

**Border zone**

![Graph showing comparison between Sham, Placebo, and CDCs]

- Sham: 1.0 ± 0.2
- Placebo: 1.2 ± 0.3
- CDCs: 0.8 ± 0.1

**p = 0.23**

**Infarct zone**

![Graph showing comparison between Sham, Placebo, and CDCs]

- Sham: 2.0 ± 0.5
- Placebo: 2.5 ± 0.4
- CDCs: 1.8 ± 0.3

**p = 0.38**
Supplementary Figure 5.

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B

**Border zone**

- Sham
- Placebo: `p=0.16`
- CDCs

**Infarct zone**

- 5M
- 7.5M: `p=0.37`
- 10M
Supplementary Table 1. Evaluation of coronary flow pre- and post-intervention by TIMI grading system in dose-escalation study.

Supplementary Table 2. Evaluation of coronary flow pre- and post-intervention by TIMI grading system in pivotal study. CDCs: cardiosphere-derived cells; TIMI: Thrombolysis in Myocardial Infarction; cTFC: corrected TIMI frame count. All groups were compared with Kruskal-Wallis test.

Supplementary Table 3. Change differences (delta values) according time points for each individual animal. BSA: body surface index; LVEF: left ventricular ejection fraction; LVEDVI: left ventricular end-diastolic volume index; LVESVI: left ventricular end-systolic volume index.

Supplementary Fig 1. Engraftment study.
Schematic of protocol to quantify short-term CDC engraftment (A). Engraftment of infused CDCs 15 minutes and 48 hours after CDC delivery (B). CDCs: cardiosphere-derived cells, AMI: acute myocardial infarction. Values are means ±SEM (standard error of the mean), *; p<0.05 between two groups.

Supplementary Fig 2. Hemorrhage evaluation in dose-escalation study.
Representative figures of hemorrhage evaluated by H-E staining at different doses of CDCs (A) and quantitative evaluation utilizing a hemorrhage scoring system (B). p value means the
result of Kruskal-Wallis test. *; p<0.05 between two groups. AU; arbitrary unit.

**Supplementary Fig 3. Corrected TIMI frame count post cell infusion in dose escalation study.**

cTFC: corrected TIMI frame count. *; p<0.05

**Supplementary Fig 4. Hemorrhage evaluation in pivotal study.**

Representative figures of hemorrhage evaluated by H-E staining in all groups (A) and quantitative evaluation utilizing a hemorrhage scoring system (B). p value means the result of Kruskal-Wallis test. AU; arbitrary unit.

**Supplementary Fig 5. Immunohistological assessment of vessel density in acute settings.**

Representative images of vessel density in the border zone assessed by immunostaining for isolectin and α-smooth muscle actin at 48hours after reperfusion in dose-escalation and pivotal study (A). Quantitative analysis of vessel density in border zone (B). p value means the result of Kruskal-Wallis test.

**REFERENCES**


