**Intracoronary Infusion of Allogeneic Mesenchymal Precursor Cells Directly After Experimental Acute Myocardial Infarction Reduces Infarct Size, Abrogates Adverse Remodeling, and Improves Cardiac Function**

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**Rationale:** Mesenchymal precursor cells (MPCs) are a specific Stro-3+ subpopulation of mesenchymal stem cells isolated from bone marrow. MPCs exert extensive cardioprotective effects, and are considered to be immune privileged.

**Objective:** This study assessed the safety, feasibility, and efficacy of intracoronary delivery of allogeneic MPCs directly after acute myocardial infarction in sheep.

**Methods and Results:** Initially, intracoronary delivery conditions were optimized in 20 sheep. These conditions were applied in a randomized study of 68 sheep with an anterior acute myocardial infarction. Coronary flow was monitored during MPC infusion, and cardiac function was assessed using invasive hemodynamics and echocardiography at baseline and during 8 weeks follow-up. Coronary flow remained within thrombolysis in myocardial infarction III definitions in all sheep during MPC infusion. Global left ventricular ejection fraction as measured by pressure–volume loop analysis deteriorated in controls to 40.7±2.6% after 8 weeks. In contrast, MPC treatment improved cardiac function to 52.8±0.7%. Echocardiography revealed significant improvement of both global and regional cardiac functions. Infarct size decreased by 40% in treated sheep, whereas infarct and border zone thickness were enhanced. Left ventricular adverse remodeling was abrogated by MPC therapy, resulting in a marked reduction of left ventricular volumes. Blood vessel density increased by >50% in the infarct and border areas. Compensatory cardiomyocyte hypertrophy was reduced in border and remote segments, accompanied by reduced collagen deposition and apoptosis. No microinfarctions in remote myocardial segments or histological abnormalities in unrelated organs were found.

**Conclusions:** Intracoronary infusion of allogeneic MPCs is safe, feasible, and markedly effective in a large animal model of acute myocardial infarction. (Circ Res. 2013;113:153-166.)

**Key Words:** cell transplantation ■ mesenchymal progenitor cell ■ mesenchymal stem cell ■ myocardial infarction ■ percutaneous coronary intervention ■ tissue therapy

Postmyocardial infarction left ventricular (LV) remodeling can lead to the clinical syndrome of heart failure, which is an increasing public health issue in the Western world. Although interventional and pharmacological therapy has improved during the past decades, the mortality and morbidity of heart failure is still considerable.1,2 Regenerative cell therapy to prevent adverse remodeling is one of the potential adjunctive therapies that has been under extensive investigation during the past few years, with promising results in phase I and II clinical trials.3

Although promising, the effects of unfractionated bone marrow mononuclear cells in these trials have been modest, and it has been suggested that mesenchymal stem cells (MSCs) or their subpopulations might be more effective.4–6 Mesenchymal precursor cells (MPCs) constitute a Stro-3 immune-selected, immature subfraction of bone marrow–derived MSCs.7 These MPCs are multipotent cells with extensive proliferative potential, and secrete numerous antiapoptotic, angiogenic factors, and growth factors. It was found that MPCs display greater cardioprotective effects than conventional MSCs that...
are selected by plastic adherence alone, which may be evoked by their potent paracrine activity, as well as more extensive multilineage differentiation potential.10–12 Interestingly, MPCs are immune privileged and can be transplanted to unrelated recipients, thereby creating the possibility of an allogeneic, off-the-shelf cell product, readily available during the acute phase of an acute myocardial infarction (AMI). Intramyocardial injection of MPCs has been shown to improve cardiac function in small and large animal models of AMI.9–12

In contrast to intramyocardial delivery, intracoronary stem cell infusion is a simple, quick, cost-effective, and reproducible delivery technique.13,14 It does not require specific infrastructure and is available in all interventional coronary catheterization laboratories, although intracoronary infusion of an off-the-shelf allogeneic stem cell product can be applied directly after revascularization of the AMI. More importantly, intracoronary infusion omits the risk of myocardial perforation and ventricular arrhythmia that has been associated with intramyocardial injection in infarcted tissue. However, intracoronary infusion of unselected and culture-expanded MSCs has previously been associated with impeded coronary flow and microinfarctions in remote myocardial segments in large animal experiments.15–18

The primary aim of the current large animal study was to assess the feasibility of intracoronary MPC infusion, and to determine the optimal infusion conditions, while carefully monitoring coronary flow, arrhythmias, and other possible cell therapy–related adverse effects. In addition, we investigated the potential efficacy of 3 different doses of MPCs using functional (pressure–volume loop [PV-loop] analysis, echocardiography) and morphological (morphometry, histology) indices during an 8-week follow-up period.

Methods

Experimental Design

A total of 88 adult sheep were used in this study. All procedures were approved by the institutional animal welfare committee (University of Utrecht, Utrecht, The Netherlands). The study was divided into 3 distinct phases. In the first phase, the maximum tolerated dose and optimal MPC infusion rate were determined in noninfarcted sheep. In the second phase, we assessed the maximum tolerated MPC dose and optimal infusion rate in animals subjected to an anterior wall AMI. The targeting potential and cell retention in the infarcted myocardial segment, as well as shedding of MPCs to remote myocardial segments and organs, were analyzed in a cell tracking substudy. Finally, in the third phase, the safety and efficacy of intracoronary infusion of 3 incremental doses of MPCs directly after AMI during an 8-week follow-up period was assessed using optimized infusion conditions (see Figure 1 for the study flow chart).

Mesenchymal Precursor Cells

MPCs used in this study were ovine Stro-3 positive bone marrow–derived cells as previously described.11,19 The cells were frozen in cryoprotectant containing 7.5% dimethyl sulfoxide and stored at −180°C in vials at a final concentration of 25 million cells per vial. Before cell infusion, MPCs were rapidly thawed, filtered through a 40-μm cell strainer, and suspended in 100 mL of lactated ringers’ solution at a final concentration of 0.5 Mill MPCs/mL (see Online Data Supplement for cell size measurements).

Phase 1: Intracoronary MPC Infusion in Noninfarcted Myocardium

A total of 12 sheep (45.2±1.5 kg) were used in phase 1. To assess the optimal infusion rate and maximum tolerated dose, naïve sheep received an intracoronary infusion of incremental doses of MPCs (25, 37.5, and 50 million) using an infusion rate of 1.25 or 2.5 million MPCs/min (Figure 1A).

A Twin Pass microcatheter (Vascular Solutions, Minneapolis, MN) was placed in the proximal left anterior descending artery (LAD), and MPCs were infused using an infusion pump (Alaris, San Diego, CA). Coronary flow was assessed regularly, and troponin I was determined at baseline and at 6 hours after cell injection (AccuTnI, Beckman Coulter, Brea, CA). After cell infusion, all animals received a subcutaneously implanted REVEAL DX event recorder (Medtronic, Minneapolis, MN) to continuously monitor for potential arrhythmias. Two days after infusion, the animals were euthanized, and the heart, lung, liver, kidney, and spleen were examined by independent pathologists.

Phase 2: Intracoronary MPC Infusion and Biodistribution After AMI

To assess the optimal infusion rate and maximum tolerated dose in AMI, intracoronary MPC infusion was performed in an anterior AMI model in 8 sheep (62.8±1.4 kg). Anterior wall AMI was induced by balloon inflation (Voyager Rx 3.0–3.5×12 mm, Abbott, Illinois) in the mid-LAD for 90 minutes. After 15 minutes of reperfusion, a Twin Pass delivery catheter was positioned in the LAD at the location of previous balloon inflation. Subsequently, 50 million MPCs were infused at a rate of 1 million MPCs/min (n=3) or 0.5 million MPCs/min (n=3). Biodistribution and myocardial retention were quantified using Indium111 labeling in 2 separate animals (see Online Data Supplement). The optimized and safe intracoronary infusion conditions that were found in phase 1 and 2 were subsequently applied in phase 3 of this study.

Phase 3: Long-Term Safety Effects and Dose Finding of Intracoronary MPC Infusion Directly After AMI

Induction of Myocardial Infarction and Infusion of MPCs

A total of 68 sheep (60.8±1.7 kg) were used in phase 3 of the study (Figure 1C). An anterior myocardial infarction was induced by LAD occlusion as described above. After reperfusion, the sheep were randomized by a blinded draw to receive an intracoronary infusion of 12.5, 25, or 37.5 million MPCs or lactated ringers’ solution (control).

The cells were infused at an infusion rate of 0.5 million MPCs/min (Figure 1A). More importantly, after infarction, the sheep were euthanized, and the heart, lung, liver, kidney, and spleen were examined by independent pathologists.

Pressure–Volume Loop Analysis

In all animals, baseline PV-loop recordings were acquired directly after MPC or placebo infusion and at 8 weeks follow-up. Also, in a random subset of animals (n=12), pre-AMI PV-loop recordings were performed to obtain reference values of PV-loop parameters of noninfarcted sheep hearts (Online Table II). Offline data analysis was performed by an investigator blinded for the treatment allocation of the individual animals (described in detail in the Online Data Supplement).

Echocardiography

In all animals, a transthoracic echocardiogram was performed at baseline and directly after the AMI, but also at 4 and 8 weeks follow-up.
LV volumes, LV ejection fraction (LVEF), regional fractional area change, and regional systolic wall thickening were analyzed offline by an operator blinded for the treatment allocation of the individual sheep (see Online Data Supplement for a detailed description).

Necropsy and Pathohistology (Long-Term Safety)
At 8 weeks follow-up, the animals were euthanized, and routine necropsy was performed to screen for any gross anatomic abnormalities in lung, kidney, spleen, liver, and gut, whereas biopsies of these organs were collected for further histological analysis. The heart was excised and prepared as described before, and stained using tetrazolium chloride (Online Figure I). Subsequently, the slices were carefully screened for microinfarctions in remote myocardial segments. Biopsies of the infarct area and infarct border zone were randomly taken and processed for further histological analysis.

Histology samples of the liver, lung, spleen, and kidney were analyzed by an independent pathology core-laboratory (Druquest International, Leeds, AL) to screen for shedding of MPCs and any remote adverse effects. A section of infarct and border zone tissue of each animal was analyzed by an independent and blinded pathologist, specialized in cardiac pathology (Erasmus University Medical Center, Rotterdam, The Netherlands) to screen for potential local adverse effects of MPC infusion.

Morphometry and Histology Parameters (Efficacy)
Photographs of tetrazolium chloride-stained slices were taken. A blinded technician calculated the percentage of total LV infarcted, and measured infarct and border zone thickness using automated image analysis software.

Collagen Content, Myocardial Salvage Index, Cardiomyocyte Size, and Cardiomyocyte Density
Collagen content, cardiomyocyte size, and cardiomyocyte density were determined using Gomorri trichrome staining.

Blood Vessel Density
Blood vessel density was determined in the border zone, remote myocardial segments, and in the infarct area. Capillary density in the border zone and remote area was assessed by isoelectin-B4 staining. Arteriolar density in the infarct area was determined using smooth muscle actin staining.

Cardiomyocyte Proliferation, Apoptosis, and Cardiac Stem Cells
The amount of proliferating cardiomyocytes was quantified using Ki-67 staining, whereas the amount of apoptotic cardiomyocytes was assessed using a terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Resident cardiac stem cells were detected by cKit staining.
Statistical Analysis

Efficacy data are depicted as placebo (n=10) versus all MPC-treated (n=20) animals, unless otherwise stated. Continuous data are presented as mean±standard error of the means. Comparisons of means (morphometry and histology) between groups were performed using a 1-way ANOVA with Bonferroni correction for multiple comparisons when applicable. Differences of PV-loop–derived and echocardiography-derived parameters between treated animals and controls were analyzed by 2-way ANOVA with repeated measures. The MPC-treated group was considered as a homogenous cohort, because ANOVA demonstrated no significant difference in relation to the MPC dose applied. A P value of ≤0.05 was considered statistically significant. The final data set and statistical analysis were audited and approved by Medical Device Consultants, Inc (MDCI, Reston, Virginia).

Results

Phase 1: MPC Infusion in Healthy Animals

Coronary flow remained within thrombolysis in myocardial infarction 3 range in all animals. However, an infusion rate of 2.5 million MPCs/min or infusion of 50 million cells resulted in an increase of troponin I levels 6 hours after infusion, and microinfarcts in the LAD territory at 2 days follow-up. In contrast, an infusion rate of 1.25 million MPCs/min permitted infusion of doses up to 37.5 million MPCs without inducing myocardial necrosis (Figure 2A and 2B). None of the animals experienced ventricular arrhythmias during the 2-day follow-up.

Analysis of hematoxylin and eosin-stained sections of all major organs rendered no differences between the sheep that received intracoronary infusion of MPCs and healthy controls. More specifically, no shedding of the allogeneic MPCs or any MPC-related acute adverse effect was detected. No acute foreign body or antiallogeneic response, defined as extensive or eosinophilic infiltrates, was found in any of the myocardial tissue specimens.

Phase 2: MPC Infusion in Infarcted Myocardium

Infusion of MPCs in the culprit artery directly after AMI at a rate of 1 million MPCs/min resulted in sluggish flow after infusion of ≥25 million MPCs (n=3; Figure 2C). After 25 million MPCs, the coronary flow rapidly declined to thrombolysis in myocardial infarction grade 1/0 and flow velocities below 10 cm/s. When applying a reduced infusion rate of 0.5 million MPCs/min, the sluggish coronary flow was only observed when the absolute dose exceeded 40 million MPCs. As a result of these findings, an infusion rate of 0.5 million MPCs/min and a maximal dose of 37.5 million MPCs were adopted in phase 3 of this study.

Phase 3: Long-Term Safety Effects and Dose Finding of Intracoronary MPC Infusion After AMI Animal Experiments

A total of 68 sheep were subjected to an anterior wall AMI by balloon occlusion in the mid-LAD for 90 minutes. Because of ventricular fibrillation refractory to defibrillation, 34 sheep died during infarct induction. The surviving animals were randomized to placebo treatment (n=12) or treatment with 12.5 (n=7), 25 (n=9), or 37.5 (n=6) million MPCs (Figure 1). Two animals...
in the control group and 2 animals in the MPC-treated group died during the 8-week follow-up (see below), resulting in 10 analyzable sheep in the control group and 20 analyzable sheep in the MPC-treated group (divided into 3 dose cohorts treated with 12.5 [n=7], 25 [n=7], or 37.5 [n=6] million MPCs). In 6 control animals and 14 MPC-treated animals, serial troponin 1 measurements were available. Both baseline (0.07±0.02 versus 0.05±0.01; P=0.89) and post-AMI (272.4±36.6 versus 297.1±29.2; P=0.66) measurements did not differ between placebo and cell-treated groups, suggesting a similar degree of injury in both groups.

Coronary Flow During and After MPC Infusion
MPC infusion was successful in all animals with thrombolysis in myocardial infarction grade 3 flow in all dose groups after MPC infusion (Figure 2D). However, infusion of 25 million MPCs led to a transient sluggish flow in 2 of 9 animals (22%), whereas infusion of 37.5 million MPCs resulted in sluggish flow in 3 of 6 (50%) animals. Sluggish flow was defined as a visual difference in the rate of opacification between the culprit artery and reference vessel (circumflex artery), whereas antegrade flow remained within thrombolysis in myocardial infarction grade 3 definitions.20 At 8-week follow-up, coronary flow had normalized in all treated animals.

Death and Ventricular Arrhythmia Analysis
Two sheep in the control group (2 of 12, 16%) and 2 sheep in the MPC-treated group (25 million MPCs; 2 of 22, 9%; P=ns) died during the 8-week follow-up period (Online Table I). Thorough analysis of the implanted event recorders demonstrated that the sheep in the control group died because of ventricular fibrillation within 12 hours after infarct induction. In the animals in the MPC group, fatal ventricular arrhythmia was excluded as the cause of death after analysis of Reveal DX data. Subsequent necropsy and histopathological examination of the heart, lung, spleen, liver, and kidney by an independent pathologist remained inconclusive about the cause of death in these MPC-treated animals. Specifically, no signs of manifest heart failure were found in lungs, liver, and spleen.

In addition to the 2 animals with a lethal arrhythmia in the control group, another control animal experienced a nonsustained ventricular tachycardia of 20 beats, 3 weeks after the index procedure.

Pressure–Volume Loop Analysis
All PV-loop–derived data can be found in Online Table II.

PV-Loop–Derived LVEF and Volumes
In control sheep, the global LVEF deteriorated from 44.2±1.5% directly after AMI to 40.7±2.6% at 8 weeks. MPC treatment markedly improved cardiac contractile function from 44.7±1.0% to 52.8±0.7% (difference between groups: +12.1%; P<0.001; Figure 3B). No clear dose–effect relationship was observed between the different dose groups.

After AMI, LV volumes were comparable between all groups. However, MPC treatment prevented cardiac remodeling at 8 weeks follow-up. LV end-systolic volume in the MPC treatment group was 68.3±4.1 mL as opposed to 102.8±4.0 mL in the control animals (−34%; P<0.001; Figure 3C). Likewise, LV end-diastolic volume (LVEDV) in the treatment group ameliorated by 16% (149.3±4.1 mL versus 178.0±8.0 mL; P<0.001) as compared with controls (Figure 3D). No significant dose–effect relationship was found between the different treatment groups.

PV-Loop–Derived, Load-Independent Indices of Systolic Function
LV contractile function is best reflected in the PV-loop derived pre- and afterload independent indices: end-systolic elastance (Ees) and preload recruitable stroke-work. Ees and preload recruitable stroke-work markedly improved during an 8-week follow-up in MPC-treated animals, as opposed to no improvement in controls. In control animals, the baseline Ees was reduced to 0.96±0.07 mm Hg.mL and remained stable at 0.89±0.05 mm Hg.mL at 8 weeks. However, in the treatment group, Ees improved from 0.99±0.06 after AMI to 1.26±0.1 mm Hg.mL (P=0.003; Figure 3A and 3E). In line with these results, preload recruitable stroke-work ameliorated to 41.6±1.9 in MPC-treated animals at 8 weeks, as opposed to 33.5±1.4 mm Hg in controls (P=0.008; Figure 3F).

PV-Loop–Derived Indices of Diastolic Function
Parameters that reflect LV stiffness, including Ees, dP/dT−, and tau, were not significantly different between control and MPC-treated groups (Online Table I). Nevertheless, there are indications that diastolic function had improved in MPC-treated animals. First, when corrected for EDV, dP/dT− improved significantly, whereas there is a clear trend toward higher end-diastolic pressures in control animals (P=0.08). Also, V0 and V10, that reflect LV end-diastolic capacitance significantly improved in MPC-treated sheep. V0 was reduced from 99.8±4.7 mL in the control group to 83.9±2.1 mL in the MPC-treated animals (P=0.001; Figure 3G), whereas the V10 was enhanced from 241±23.5 mL in the control group to 213±8.2 mL in the MPC-treated group (P=0.047; Figure 3H).

Echocardiography
All echocardiography measures can be found in the Online Table III. Global LV function, as depicted by LVEF, was comparable between groups before and directly after AMI (Figure 4A–4C). After AMI, LVEF decreased by 20% in both treatment and placebo groups. In the control group, LVEF gradually deteriorated further from 43.1±1.2% after AMI to 37.3±1.9% at the 8-week follow-up. In contrast, LVEF improved by +21% to 47.7±1.2% in sheep treated with MPCs (P=0.001; Figure 4A), when compared with placebo animals, thereby corroborating the PV-loop data.

Echocardiography demonstrated that both LVEDV and LV end-systolic volume were comparable at baseline and directly after AMI. Importantly, it confirmed PV-loop derived volumes at baseline and the improvement at 8 weeks follow-up, as shown in Figure 3. LVEDV and LV end-systolic volume deteriorated in both groups during the 8-week follow-up period, but the increase in the placebo group was significantly greater than in the MPC-treated group. LVEDV increased to 182.9±22.5 mL in control animals, and was reduced by 25% to 136.2±12.0 mL in the treatment group (P=0.037). LV end-systolic volume improved by almost 40% from 115.8±16.5 mL in placebo-treated animals to 71.3±6.9 mL in MPC-treated animals (P=0.042).
Regional function improved by MPC therapy, because fractional area change in the apex was 39% higher in cell-treated animals as compared with controls (41.4±2.7% versus 29.8±2.0%; \( P = 0.027 \); Figure 5A), and fractional area change was 30% higher in the midventricle (46.7±1.7 versus 35.8±2.6%; \( P = 0.007 \); Figure 5B). Fractional area change in the basal segments of the heart did not differ significantly between control and treated animals (Figure 5C; \( P = 0.57 \)). No clear dose–effect relationship was found, and all 3 doses seemed to be equally effective.

Also, regional contractility improved by MPC therapy. Systolic wall thickening was severely impaired in the apical and midventricular anterior and anteroseptal wall segments directly after AMI, whereas compensatory hypercontractility was present in a contralateral remote myocardial segment (Figure 5). Systolic wall thickening in the anteroseptal segment of the apex improved from 0.5±0.6% in control animals to 23.7±4.2% in treated animals (\( P = 0.003 \); Figure 5D) and in the anterior wall from 2.7±4.3% to 24.9±2.4% (\( P < 0.001 \); Figure 5E). Both infarcted segments also markedly improved at the midventricular level (anteroseptal segment; co 9.6±5.5% versus MPC treated 39.1±1.8%, \( P < 0.001 \); anterior wall; co 13.8±3.6% versus MPC treated 34.7±1.9%, \( P < 0.001 \); Figure 5F and 5G). No significant difference between both groups was found in the contralateral myocardial segment (\( P = 0.32 \) and 0.22, respectively; Figure 5H and 5I).

Figure 3. Pressure–volume loop (PV-loop) analysis. A, Typical examples of PV-loops of individual animals in the 4 evaluated groups. The gray loop represents a normal PV-loop of a noninfarcted sheep heart, whereas the black loop represents the PV-relation briefly after an acute myocardial infarction (AMI). After 8 weeks (red loop), the PV-loop in the control animal shows a rightward shift, indicating increased volumes, further decline of the end-systolic elastance (\( E_s \)), and increased end-diastolic pressure (filling pressure). In mesenchymal precursor cell (MPC)-treated animals, left ventricular dimensions were preserved, whereas \( E_s \) returned to near baseline levels. B, Left ventricular (LV) ejection fraction further deteriorated in control animals, but was enhanced by >30% after MPC therapy. C and D, LV volumes increased in the control group, indicative of LV remodeling. This remodeling process was abrogated by MPC therapy. E and F, Preload and afterload independent parameters of myocardial contractility, \( E_s \) and preload recruitable stroke-work (PRSW), were enhanced in MPC-treated sheep as compared with controls. G and H, \( V_0 \) and \( V_30 \) are both points on the end-diastolic pressure–volume relation and represent diastolic function and capacitance. ns indicates nonsignificant. *\( P \leq 0.05 \); **\( P \leq 0.01 \); ***\( P \leq 0.001 \).

Necropsy and Histopathology Analysis of Tissue Samples

During autopsy and macroscopic analysis, no signs of gross anatomic malformations, neoplasms, or angiomas were detected in the heart, gut, liver, lungs, kidneys, and spleen. This was confirmed by histological analysis by independent pathologists. In the tetrazolium chloride-stained slices of the heart, no signs of microinfarctions in remote myocardial segments were found.

Infarct Size and Morphometry

The percentage of LV infarcted in control animals measured 18.4±1.5% in placebo controls, and improved by 33% to 12.0±0.7% in MPC-treated animals (Figure 6A; \( P = 0.001 \)). Also, the average infarct wall thickness in the midventricle was enhanced by 25% in treated animals as compared with...
control animals (6.4±0.2 versus 8.0±0.3 mm; \( P < 0.001 \); Figure 6C). In control animals, the average border zone thickness was 8.5±0.48 mm in the midventricle, whereas it improved to 10.5±0.5 mm \((P = 0.011)\) in MPC-treated animals, respectively (Figure 4D).

**Histology**

**Collagen Content**

MPCs significantly reduced extracellular matrix deposition in all myocardial areas. Collagen content in the border zone decreased from 16.5±2.1% in the control group to 7.4±0.7% \((P < 0.001;\) Figure 7A) in the treatment group, whereas collagen content in remote myocardial segments decreased from 2.1±0.4% to 1.0±0.2% \((P = 0.001;\) Figure 7B).

Interestingly, also in the infarct area, the collagen content was significantly reduced in MPC-treated animals as compared with controls (Figure 7C). This decrease in collagen content favored the amount of viable myocardium in infarct specimens, suggesting myocardial salvage. This was represented by a marked improvement in the myocardial salvage index from 0.29±0.06 in controls to 1.30±0.20 in MPC-treated sheep \((P = 0.002;\) Figure 7D). No clear dose–effect relationship was present in the collagen deposition in all segments.

**Blood Vessel Density, Cardiomyocyte Size, and Cardiomyocyte Density**

The number of capillaries in the border zone was enhanced by 58% from 1196±87 capillaries/mm\(^2\) in the control group to 1894±105 capillaries/mm\(^2\) in the MPC-treated sheep \((P < 0.001;\) Figure 4).
Figure 8A). Although not statistically significant, there seemed to be an incremental dose–effect relationship in capillary density between the dose groups (12.5 mol/L: 1704±144; 25 mol/L: 1953±232; 37.5 mol/L: 2046±144 capillaries/mm²). The higher capillary density in the border zone resulted in a 35% increase of the capillary-to-cardiomyocyte ratio. In MPC-treated animals, each cardiomyocyte was supported by 1.39±0.15 capillaries on average, whereas this was reduced to only 1.01±0.11 in control animals (Figure 8B; \( P = 0.012 \)). In contrast, no difference between groups was found in the capillary-to-cardiomyocyte ratio in remote myocardial segments (Figure 8B; controls: 1.81±0.12 capillaries/cardiacmyocyte versus MPC-treated: 1.73±0.19 capillaries/cardiacmyocyte; \( P = 0.856 \)).

The arteriolar density in the infarct area was remarkably enhanced by MPC therapy. In the treatment group, arteriolar density doubled as compared with the control group (49.2±4.1 versus 21.7±4.0 arterioles/mm²; \( P < 0.001 \); Figure 8C).

Post-AMI compensatory cardiomyocyte hypertrophy in border zone and remote areas was more pronounced in the control group as compared with the MPC-treated group (49.2±4.1 versus 21.7±4.0 arterioles/mm²; \( P < 0.001 \); Figure 8C).

Apoptosis, Cardiomyocyte Proliferation, and Resident Cardiac Stem Cells

Apoptotic, terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cardiomyocytes constituted 1.31±0.15% of total cardiomyocytes in the border zone of control animals and were reduced by 40% in MPC-treated animals to 0.77±0.12% (Figure 8G; \( P = 0.008 \)). MPC therapy also had a favorable effect in remote myocardial segments by lowering the percentage of apoptotic cardiomyocytes from 0.63±0.12% in controls to 0.43±0.03% in MPC-treated animals (Figure 8H; \( P = 0.037 \)).

This effect on programmed cell death was accompanied by a small, but significant, increase in proliferating cardiomyocytes in the infarct border zone, but not in remote segments (Figure 8I and 8J). In MPC-treated sheep, 1.38±0.08‰ of cardiomyocytes were positive for Ki-67, as opposed to 0.97±0.08‰ in placebo controls (\( P = 0.02 \)).

Resident cardiac stem cells, defined as cKit+ cells, were rarely found in both remote segments and in the infarct border zone (0.071±0.012‰ in controls versus 0.098±0.022‰ in treated animals; \( P = 0.30 \)), and were not significantly increased by MPC therapy (Figure 8K).

Discussion

In the current study, we investigated a primitive subpopulation of bone marrow–derived MSCs. These Stro-3+ cells were previously shown to possess potent cardioprotective
and immunomodulatory properties in vitro and in vivo, and can be given to patients in an allogeneic setting. We found that, when certain conditions are adopted, intracoronary infusion of these MPC can be performed safely after AMI without adverse effects, impeding coronary flow, or micro-infarctions in remote myocardial segments. Moreover, we demonstrate that intracoronary delivery of MPC prevents LV remodeling and improves residual cardiac function. The results of this study suggest that these effects are evoked by myocardial salvage and subsequent reduction of infarct size, accompanied by induced angiogenesis and reduced myocardial fibrosis.

Previous Experience With Intracoronary Infusion of MSCs

Previous studies showed that intracoronary infusion of nonselected MSCs was associated with microvascular obstruction, coronary flow reduction, and myocardial infarctions attributable to capillary plugging.15–18 The prominent microvascular obstruction that was found in these previous studies might be explained by several factors. First, the size of nonselected MSCs progressively increases during cell culturing and higher passages to well >30 to 50 micrometer.21 In contrast, MPCs comprise an immature subpopulation of MSCs with a median diameter of only 13 micrometer, even when expanded in cell culture (Figure 8L; Online Data Supplement). Because the diameter of capillaries does not exceed 6 to 10 micrometer, we believe that this small cell size facilitates intracoronary infusion.

Second, in previous studies, higher absolute doses of MSC were used, whereas relatively higher infusion rates were adopted than in the current study. For example, the study of Perin et al15 infused 100 million MSCs at a rate of 1 million cells per minute, whereas Freyman et al17 infused 50 million cells at a rate of 1.5 million cells per minute. In these studies, microvascular obstruction and no-flow phenomena are described. On the contrary, infusion of lower cell numbers did not hamper coronary flow in previous experiments. Valina et al22 infused only 2 million MSCs directly after AMI, and Suzuki et al23 infused 15 million MSCs per coronary artery in hibernating myocardium, both without any flow-related side-effects. Also, in a study by Johnston et al24, infusion of 10 million cardiosphere-derived cells (20 micrometer in diameter) after AMI was deemed safe, whereas 25 million cells or more caused significant infarctions.

Safety and Targeting Efficiency of Intracoronary MPC Infusion

After several pilot experiments divided into 2 separate phases, we found that, after AMI, a low infusion rate of only 0.5 million MPCs/min permitted intracoronary infusion of 50 million cells without permanently compromising coronary flow, whereas higher infusion rates decreased the maximum tolerated dose. Interestingly, a marked difference between the maximally tolerated dose and infusion rates in animals with or without AMI was noted. This might be associated with increased vascular adhesion of the cells caused by increased expression of chemokines and cell adhesion factors by the activated endothelium after the ischemic insult.25
Figure 8. Blood vessel density, cardiomyocyte (CMC) size, apoptosis, proliferation, and cardiac stem cells. A, Capillary density was assessed in the border zone, revealing increased capillary densities in mesenchymal precursor cell (MPC)-treated sheep. B, The capillary-to-cardiomyocyte ratio was only enhanced in the perfusion territory of the culprit artery of MPC-treated sheep as compared with no change in placebo controls, or in remote myocardial segments. C, In the infarct area, a doubling of arteriolar density of MPC-treated animals was observed. D and E, Cardiomyocyte hypertrophy was markedly reduced in the border zones, as well as in remote myocardial segments of MPC-treated animals, when compared with controls. F, This was confirmed by an increase in cardiomyocyte nuclear density in both border zone and remote areas, and is suggestive of delayed or abrogated adverse remodeling that typically precedes clinical heart failure. Together with the profound effect on cardiac function, it also strongly suggests cardiomyocyte regeneration. G and H, MPC therapy reduced cardiomyocyte apoptosis in both border and remote myocardial segments, corroborating reduced adverse remodeling in MPC-treated sheep hearts. I and J, MPC therapy stimulated cardiomyocytes in the infarct border zone to reenter the cell cycle, thereby increasing the number of proliferating cells and inducing endogenous repair. The top picture bordering the graph shows Ki67-positive cells in the gut that served as positive control. The bottom picture shows a Ki67-positive nucleus of a cardiomyocyte. K, cKit staining revealed that the amount of resident cardiac stem cells did not increase in MPC-treated animals in both border and remote areas. The top picture bordering the graph shows cKit-positive cells in a perivascular area of the myocardium (cardiomyocytes are red). L, Microphotograph of MPCs in suspension. MPCs are considerably smaller than nonimmune-selected, cultured mesenchymal stem cells that reach sizes of >30 µm. M indicates million; and ns, nonsignificant. *P≤0.05; **P≤0.01; ***P≤0.001.
We hypothesize that a low infusion rate might enable the MPCs to either pass through the capillary bed or to transmigrate into the perivascular tissue without aggregation or capillary occlusion. Indeed, the nuclear imaging retention substudy in 2 animals revealed that a significant number of MPCs still resided in the heart 2 hours after intracoronary infusion, whereas epicardial coronary flow remained normal (see Online Data Supplement). Importantly, no microinfarctions, or shedding of MPCs to, remote myocardial segments were detected by macroscopic inspection, microscopic analysis, and nuclear imaging techniques. This demonstrates that intracoronary infusion of culture-expanded MPCs is feasible and can be performed safely. Furthermore, MPC therapy did not have any proarrhythmogenic effect. On the contrary, the MPC-treated group showed a trend toward a reduction of ventricular arrhythmias. This might be correlated with the reduction of scar size and improved myocardial perfusion in MPC-treated animals.

Also, no signs of tumorous growth or other focal abnormalities were detected in tissue samples of all major organs or sections of the infarct area by independent and blinded core-laboratory histological analyses. These findings provide additional safety data, because shedding of the cells did not result in significant side-effects, engraftment or aberrant growth in the infused area or remote organs.

Proposed Working Mechanism of MPC Therapy in AMI

The predominant working mechanism of MPC therapy in cardiovascular disease is generally considered to be through paracrine actions of the cells, because long-term engraftment and transdifferentiation into cardiomyocytes of MPCs were found to be unlikely in previous studies, and cannot account for the profound beneficial effect that has been found in numerous studies. Indeed, MPCs are known to secrete significant amounts of relevant growth and angiogenic factors as stromal cell–derived factor-1, hepatocyte growth factor-1, insulin-like growth factor-1, vascular endothelial growth factor, and interleukin-6. Importantly, the release of these factors exceeds the paracrine abilities of nonselected MSCs, resulting in better cardioprotective properties of MPCs when compared with MSCs. Although actual cell engraftment and possible transdifferentiation of MPCs into cardiomyocytes was not determined in the current study, we believe that it provides insightful data on the regenerative potential and working mechanism of post-AMI cell therapy using allogeneic MPCs.

Cardiomyocyte Salvage and Reduced Adverse Remodeling

Because the cells were administered directly after reperfusion of the AMI, we hypothesize that the therapeutic effect of the MPCs is mainly exerted through the release of antiapoptotic and prosurvival factors, thereby ascertaining cardiomyocyte salvage. In addition, the profound immunomodulatory actions of MPCs may preserve myocardial tissue and contribute to effective tissue healing with limiting scar tissue formation by ameliorating reperfusion injury or attenuating oxidative stress. The presumed efficacy of stem cell therapy within the first hours or days after an AMI was also suggested in 2 studies that used intracoronary delivery of MSC-like stem cells isolated from adipose tissue.

The reduction of infarct size in MPC-treated animals might have resulted in alleviated LV wall stress and reduced neurohumoral activation. This may then ultimately prevent interstitial fibrosis and compensatory cardiomyocyte hypertrophy in the noninfarcted myocardium and, in the long term, LV dilation. Moreover, in control animals, more apoptotic cardiomyocytes were found in both infarct-related and remote segments, which is a strong indication of ongoing adverse remodeling. Indeed, the placebo-treated animals exhibited increased filling pressures and impaired filling rates, a rightward shift of the PV-relation (ie, increased volumes), and more myocardial fibrosis and cardiomyocyte hypertrophy when compared with MPC-treated animals. These parameters are all part of the structural remodeling process that is generally progressive and precedes the clinical syndrome of congestive heart failure with poor prognosis.

Cardiomyocyte Proliferation and Resident Cardiac Stem Cells

Recent studies have shown that also the postnatal heart contains resident stem cells. Delivery of MSCs to infarcted or hibernating myocardium can regenerate myocardium and improve cardiac function by stimulating these resident cardiac stem cells and cardiomyocytes to (re)enter the cell cycle, thereby initiating cardiomyocyte generation or proliferation. In our study, we found a marked difference in cardiomyocyte number and cardiomyocyte size in infarct border, as well as in remote myocardial segments of MPC-treated animals. We believe that this difference in part can be explained by initial myocardial salvage, resulting in subsequent reduction of compensatory cardiomyocyte hypertrophy and apoptosis, and eventually in abrogated adverse remodeling. However, the mere size of the effect on contractile function and cardiomyocyte number, as well as the fact that also remote areas participate, may suggest syngeneic therapeutic working mechanisms induced by the infused MPCs. For instance, the current results indicate that in MPC-treated hearts, increased numbers of cardiomyocytes are in a proliferative state. These proliferating cardiomyocytes might comprise mature proliferating cardiomyocytes that have re-entered the cell cycle, but can also represent the end stage of differentiating cardiac stem cells. Although we found no significant effect on cardiomyocyte proliferation in remote segments after 8 weeks, based on evidence in previous preclinical studies, we hypothesize that this is primarily because of the fact that this effect on cardiomyocyte proliferation may have been transient, and the current time point was too late to capture it. We pose that cardiac stem cell niches, which are primarily located in the apex and around the atria of the adult heart, might have been activated by the infused MPCs, as was also suggested by Suzuki et al. Because the apical stem cell niche was probably depleted by ischemic damage, we hypothesize that cardiac stem cells originating from peri-atrial tissue might have migrated from the base of the heart to the apical, damaged area, thereby eventually not only regenerating the peri-infarct region, but also repopulating remote remote segments. It is plausible that increased cardiomyocyte
number in basal remote segments and enhanced proliferation in the apical peri-infarct region are both late effects of this time-dependent cardiac stem cell activation and migration from base to apex. It should be noted, however, that in contrast to previous studies, we found no clear difference in the amount of cardiac cKit+ stem cells between treated and control animals, which again might be explained by the longer follow-up period of the current study. Also, the amount of resident cardiac stem cells in sheep myocardium was rather low, possibly caused by the fact that in previous studies, mice and juvenile pigs were used, whereas the current study was performed in adult sheep. We hypothesize that rodents and juvenile pigs may have more resident cardiac stem cells than adult animals, although direct comparative study data are still lacking. Importantly, the fact that MPC therapy also beneficially affects remote regions, which constitute >80% of the injured heart, might explain the profound effect on remodeling, and both global and regional cardiac contractile function that was found in MPC-treated animals.

Induced Neocapillary and Arteriole Formation
Besides the effect on infarct size, remodeling, and cardiomyocyte proliferation, we also found a marked increase in neocapillary and arteriole densities in the infarct border zone and infarct area of MPC-treated animals. This increase in blood vessel density in the perfusion territory of the culprit artery suggests a proangiogenic potential of MPC therapy and is consistent with previous studies. Although we have not directly assessed myocardial perfusion using functional testing, these histological data suggest improved myocardial perfusion and, therefore, oxygen and nutrient delivery in the (peri-) infarct region. This might in part explain the enhanced regional cardiac function and contractility that were found in this study.

Previous Experience With MPCs in AMI
In previous large animal studies that assessed the effect of MPC transplantation after AMI, MPCs were injected intramyocardially. MPC transplantation was shown to attenuate LV remodeling and improve cardiac function by enhancing vascular densities, and altering collagen dynamics. These studies also revealed that the low-dose groups (≤75 million MPCs) performed better than the groups that received higher doses (>200 million MPCs), suggesting an inverse dose–response relationship and a therapeutic threshold of MPC therapy. Accordingly, we found a marked therapeutic effect on both regional and global cardiac functions at a relatively low-dose range, although no clear correlation was found between efficacy and the cell dose applied. We speculate that higher doses of MPCs may be effective, yet also lead to more microvascular obstruction that may counteract the therapeutic effect, whereas even lower doses might still be effective. In another study, See et al. for the first time compared MPCs with conventional MSCs, thereby showing the extensive cardioprotective and proangiogenic paracrine capacities of MPCs that exceed the paracrine capacities of nonselected MSCs. They showed that paracrine actions were likely the predominant working mechanism of MPC therapy. The current study not only elaborates on these findings, but also adds to our understanding of the working mechanism of MPC therapy. We not only confirm that MPCs exert cardioprotective effects, reduce fibrosis, and increase blood vessel densities in infarct and infarct border zone, but also, for the first time, show effects on cardiomyocyte proliferation with hints of stem cell activation. By determining optimal intracoronary delivery conditions in a relevant large animal model, we paved the way for clinical studies in the near future that use a protocol based on the results of the current study.

Advantages of Allogeneic Cell Therapy
An allogeneic, off-the-shelf, cell therapy product, originally derived from a young and healthy donor, has important advantages. It renders a laborious and potentially dangerous bone marrow puncture, as well as the culturing steps in clean room facilities, unnecessary. In addition, the stem cell line ensures adequate quality control with inherent batch-to-batch consistency. Also, a negative correlation was found between the amount and functionality of progenitor cells, and age and cardiovascular risk factors. This would make the use of allogeneic MPCs in the typically elderly, cardiovascular patient population preferable over autologous cells. More importantly, the cell therapy can be initiated directly after the revascularization of the AMI, thereby maximally using the antiapoptotic and immunomodulatory capacities of the cells. Finally, intracoronary delivery of an off-the-shelf cell product can be easily performed in any interventional catheterization laboratory in the world, without the need for specific infrastructure or cell delivery techniques.

Clinical Experience and Prospects
Recently, the results from a clinical, phase Ia study, assessing the effect of intramyocardial injections of allogeneic MPCs in 60 patients with heart failure, were presented. Allogeneic MPC injections up to a dose of 150 million cells were shown to be safe and feasible without a clinically significant antiallogeneic immune response. More importantly, major adverse cardio and cerebrovascular events rate, cardiac mortality, and composite end points for heart failure were markedly decreased at 12-month clinical follow-up. This study resulted in the preparations of a phase III study, analyzing the therapeutic effect of MPC therapy via intramyocardial injections in 1700 patients with congestive heart failure. Likewise, the robust effects of MPC therapy in the current large animal AMI study have led to the design of a multicenter, phase IIa/b, double blind, randomized, and placebo-controlled clinical trial. The Allogeneic Mesenchymal precursor cell Infusion in myoCardial Infarction (AMICI) trial, in which European, Australian, and US sites will participate, is aimed to prove safety, feasibility, and efficacy of MPC therapy in a minimum of 225 patients with ST-elevation AMI and will start enrollment in Q1 of 2013.

Limitations
Although the current randomized study was performed by blinded operators, and histopathology, PV-loop, echocardiography, and histology data were analyzed by blinded pathologists or technicians, it also has some limitations. First, the use of cardiac MRI would have supplied additional data on baseline infarct size, and might have rendered slightly more reliable analysis of LV volume. Because of logistical reasons
these data are lacking, but we are confident that the combined echocardiography and PV-loop analysis provide adequate and corroborating functional data on both regional and global LV functions. Also, we used a nonatherosclerotic animal model without significant thrombus burden causing the AMI. In the real world, the dynamics of MPCs after intracoronary infusion in patients with atherosclerotic and microvascular disease might be different and result in earlier flow-related effects. Hence, in the forthcoming AMICI trial, the highest dose tested in our preclinical study was omitted. Also, to prevent further loss of animals because of ventricular arrhythmias, all sheep were premedicated with amiodarone. Amiodarone treatment was continued throughout the 8-week follow-up, which is different from the real life AMI treatment and might have clouded arrhythmia analysis.

Ideally, a control group of cultured, nonimmune-selected MSCs should have been part of this study. However, previous studies have shown that intracoronary infusion of comparable amounts of nonselected MSCs would have resulted in microvascular obstruction and no-flow phenomena, which would have resulted in a substantial loss of animals. Also, the efficacy of MSCs has been established before,35 which makes the addition of an extra group to this already large study obsolete.

Conclusion
Intracoronary infusion of allogeneic primitive MPCs directly after an AMI is feasible and safe when certain conditions are adopted. It reduces infarct size and prevents subsequent adverse cardiac remodeling by cardiomyocyte salvage and angiogenesis, thereby preserving cardiac function and dimensions. The findings of this study might extend the possible application of these cells from specialized cell therapy centers to virtually any Interventional catheterization laboratory in the world. Because MPCs can be applied as an off-the-shelf product to all AMI patients, the target patient population is considerable, with more than 800 primary percutaneous coronary interventions per million inhabitants in Europe alone.36

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Disclosures
S. Itescu is the co-founder and CEO of Mesoblast Ltd. The other authors report no conflicts.

References
**Novelty and Significance**

**What Is Known?**

- Intracoronary delivery of mesenchymal stem cells has been complicated by vascular plugging, no-flow phenomena, and the occurrence of myocardial infarctions.
- Mesenchymal precursor cells (MPCs) are a Stro-3+, immune-selected subpopulation of mesenchymal stem cells that exceed the cardioprotective and proangiogenic properties of mesenchymal stem cells.
- Intramyocardial injection of allogenic MPCs improves cardiac function in animal models of acute myocardial infarction.

**What New Information Does This Article Contribute?**

- When certain conditions are adopted, intracoronary infusion of MPCs is safe and feasible.
- Intracoronary infusion of MPCs directly after acute myocardial infarction reduces infarct size, decreases adverse remodeling, and improves regional and global left ventricular functions.
- Functional improvement is evoked by cardiomyocyte (CMC) salvage, decreased CMC apoptosis, stimulation of CMC proliferation or cardiac stem cell activation, enhanced angiogenesis, and reduced fibrosis.

Clinical trials using bone marrow–derived mononuclear cells show modest benefits in patients with acute myocardial infarction. Cell therapy using MPCs might be more effective because of their more pronounced cardioprotective properties. However, intracoronary infusion of therapeutic amounts of mesenchymal stem cells has been complicated by vascular plugging, rendering these cells unsuitable for this delivery technique. We found that intracoronary infusion of MPCs can be performed safely when certain conditions are adopted. Moreover, intracoronary infusion of MPCs directly after acute myocardial infarction markedly reduced infarct size and adverse remodeling, and preserved LV function. This is achieved by CMC salvage, decreased CMC apoptosis, enhanced angiogenesis, and reduced fibrosis, but also stimulation of CMC proliferation or cardiac stem cell activation. These findings could inform future clinical trials with MPCs.
Intracoronary Infusion of Allogeneic Mesenchymal Precursor Cells Directly After Experimental Acute Myocardial Infarction Reduces Infarct Size, Abrogates Adverse Remodeling, and Improves Cardiac Function

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Materials and Methods

Medication
All sheep were pre-treated with dual anti-platelet therapy (acetylsalicylic acid (Centrafarm, Etten-Leur, the Netherlands) 80 mg qd, clopidogrel (Sanofi-Aventis, Paris, France) 75 mg qd) and amiodarone (Centrafarm, Etten-Leur, the Netherlands) 400 mg qd for ten days prior to the index procedure. Before infarct induction, an intravenous bolus of 10 mg of metoprolol (AstraZeneca, London, United Kingdom) and 10,000 IU heparin (Leo pharma, Ballerup, Denmark) were administered. All sheep received eptifibatide (Merck, Whitehouse Station, USA; bolus of 180 μg/kg and 2 μg/kg/min) during the entire procedure.

Measurement of cell diameter of mesenchymal precursor cells
A vial of 25 million MPC was rapidly thawed, resuspended in a total volume of 25 mL of lactated Ringer’s solution and washed twice. Upon the final resuspension step, cells were filtered through a 40 micron cell strainer to obtain a single cell suspension. Subsequently, several cytosmears were made, which were left to air dry. The cytosmears were fixed by submersion in methanol for 15 seconds, after which a common H&E staining was performed. The cytosmears were examined at 1000x magnification and pictures were taken. The diameter of a total of 500 MPC was measured using a routine in automated quantification software as mentioned elsewhere, and the mean and median size were calculated.

Phase 1 – Intracoronary MPC infusion in non-infarcted myocardium
A total of 12 sheep were used in phase 1. To assess the optimal infusion rate and maximum tolerated dose, naïve sheep received an intracoronary infusion of incremental doses of MPC (25, 37.5 and 50 million) using an infusion rate of 1.25 or 2.5 million MPC/min (Figure 1A). A Twin Pass® micro-catheter (Vascular Solutions, Minneapolis, USA) was placed in the proximal LAD and MPC were infused using an infusion pump (Alaris, San Diego, USA). Coronary flow was assessed by visual estimation of TIMI coronary flow at baseline, every five minutes during MPC infusion, and directly following MPC infusion, to evaluate microvascular obstruction as suggested by reduced antegrade coronary flow. Troponin I (TnI) was determined at baseline, 6 and 24 hours post cell injection (AccuTnI, Beckman Coulter, Brea, USA). TnI levels above 0.1 microgram/L were considered to be an indication of significant cardiomyocyte necrosis due to microvascular obstruction by the infused MPC. After cell infusion, all animals received a subcutaneously implanted REVEAL DX® event recorder (Medtronic, Minneapolis, USA) to continuously monitor for potential arrhythmias. After 48 hours, the animals were sacrificed, the hearts excised, and sectioned into five bread-loafed slices of 8-10 mm from apex to base. The sections were stained with 2,3,5-Triphenyltetrazolium chloride (TTC) to visualize (micro-)infarctions. Samples were taken from the inferolateral wall (remote myocardial segment) and anteroseptal wall (target area), as well as from lung, liver, kidney and spleen for histological analysis by an independent pathologist (Erasmus University Medical Center, Rotterdam, The Netherlands) blinded to the individual treatment of the animals.

Phase 2 – Intracoronary MPC infusion and bio-distribution following AMI
To assess the optimal infusion rate and maximum tolerated dose in AMI, intracoronary MPC infusion was performed in an anterior AMI model in 8 sheep. Coronary flow was assessed by visual estimation of TIMI coronary flow, and quantified by intracoronary Doppler flow analysis using a Doppler flow wire (Combowire®, Volcano, San Diego, USA) positioned between the first and second diagonal branch of the LAD, and expressed as the average peak velocity (APV; cm/sec; figure 1B). Bio-distribution and myocardial retention was quantified using Indium111 labeling in two separate animals (see below). Anterior wall AMI was induced by balloon inflation (Voyager Rx 3.0-3.5x12 mm, Abbott, Illinois, USA) in the mid LAD for 90 minutes. After 15 minutes of reperfusion, a Twin Pass® delivery catheter was positioned in the LAD at the location of prior balloon inflation.
Subsequently, 50 million MPC were infused at a rate of 1 million MPC/min (n=3) or 0.5 million MPC/min (n=3). The maximum tolerated dose of MPC was assessed by repeated Doppler flow measurements after infusion of every 5 million cells. In addition, TIMI flow was determined after infusion of every 10 million MPC. The optimized and safe intracoronary infusion conditions were subsequently applied in phase 3 of this study.

Phase 3 – Long-term safety effects and dose finding of intracoronary MPC infusion after AMI

**Induction of myocardial infarction and infusion of MPC**

A total of 68 sheep were used in phase 3 of the study (figure 1C). An anterior myocardial infarction was induced by LAD occlusion as described before. After reperfusion, the sheep were randomized by a blinded draw to receive an intracoronary infusion of 12.5, 25, or 37.5 million MPC or LR (control). The cells were infused via a Twin Pass® delivery catheter at an infusion rate of 0.5 million MPC/min. Coronary flow was assessed by coronary angiography before cell infusion, and every 15 minutes during cell infusion. Coronary angiographies were scored by a blinded interventional cardiologist at Thoraxcenter of the Erasmus University Medical Center in Rotterdam, to prevent bias. After cell infusion, a subcutaneous event recorder was implanted to monitor for ventricular arrhythmias during the 8 week follow-up. Eight weeks following AMI and MPC infusion, coronary angiography and TIMI flow grade assessments were performed and analyzed by independent and blinded investigators.

**Nuclear labeling and imaging**

MPC were labeled with Indium¹¹¹ at 37°C for 20 minutes (20MBq; GE Healthcare, Pittsburgh, USA). After incubation, cells were washed three times with HANKS buffer (Invitrogen, Carlsbad, USA) and Indium¹¹¹ uptake efficiency was measured with a dose calibrator (Veenstra, Joure, the Netherlands), whereas cell viability was assessed by trypan-blue counting. A total of 37.5 million MPC were infused following reperfusion of the culprit vessel at a rate of 0.5 million MPC/min. Animals were sacrificed two hours after MPC infusion and heart, lungs, liver, spleen and kidneys were excised. Subsequently, the organs, urinary catheter system and the infusion system (syringes, tubing, catheters) were scanned using a dual-head gamma camera (Philips, Best, the Netherlands) to quantify MPC bio distribution (acquisition of 5 minutes, 256x256 projection matrix). Bio distribution and retention were determined using dedicated software (Pegasys, Philips, Best, the Netherlands) and expressed as percentage of the injected dose per organ.

**Pressure–volume loop calibration, parameters and analysis**

The volume was calibrated by thermodilution and hypertonic saline dilution as previously described.² ³ PV-loop measurements were performed at baseline and at eight-week follow-up and analyzed using customized software (Conduct NT 2.18, CD Leycom, Zoetermeer, The Netherlands). All values are based on the analysis of ten consecutive beats of sinus rhythm. End-systolic elastance (Eₚₑₛ) was defined as the slope of the end-systolic pressure–volume relation (ESPVR). The Eₚₑₛ was calculated using a single-beat estimation method as previously described.⁴ ⁵ The V₀ of the end-diastolic pressure–volume relation (EDPVR), which represents the unstretched volume of the LV, was calculated using the following formula: (0.6-0.006*end diastolic pressure (EDP))*end-diastolic volume (EDV)).⁶ ⁷ The V₃₀ is the theoretic point on the EDPVR where the pressure is 30 mmHg and was calculated using the following formula: V₀ + (EDV- V₀)/(EDP/27.8)(¹/²).⁶ ⁷ End-diastolic stiffness (Eₑ₀) was calculated as EDV/EDP and preload-recruitable stroke-work (PRSW) as stroke-work/EDV.⁸

**Echocardiography**

In all animals, a transthoracic echocardiogram (TTE) was made before the AMI and directly following the AMI, and at four and eight weeks follow up. Two-dimensional grey scale images at a frame rate of 60-90 frames/s were obtained from a parasternal position with a Philips iE33, equipped with a broadband S5-1 transducer (Philips Healthcare, Eindhoven, The Netherlands). Short axis views were recorded at three different levels (basal, mid ventricular...
and apical) and three consecutive cardiac cycles were acquired. These images were transferred to an Image Arena 4.1 (Tomtec Imaging Systems, Unterschleissheim, Germany) work station for offline analysis. The analysis of echocardiography data was performed by an independent operator, who was blinded for the treatment allocation of the sheep.

The endocardial border was traced at end-diastole and end-systole at each level. LV volumes were calculated using modified Simpson’s rule: LV end diastolic volume (LVEDV)= \((A_{BED} \times L/3) + (A_{MID} + A_{APE})/2 \times L/3 + 1/3(A_{APE}) \times L/3\); LV end systolic volume (LVESV)= \((A_{RES} \times L/3) + (A_{MID} + A_{APE})/2 \times L/3 + 1/3(A_{APE}) \times L/3\), in which \(A_b\) is the area at basal level, whereas \(A_m\) and \(A_a\) are the areas at mid and apical level respectively. \(L\) is defined as the length of the ventricle from apex to base, and was set at 10 cm at baseline, based on cadaver measurements. \(L\) at 8 week follow up was measured by counting up the thicknesses of all post-mortem slices (see supplemental figure 1), and \(L\) at 4 week follow up was estimated by calculating the mean between baseline and 8 week follow up per animal. LVEF was calculated as follows: \(((LVEDV-LVESV)/LVEDV) \times 100\). These estimations of LV volumes and EF were shown to correspond very well with radionuclide measurement techniques.\(^9\)

Regional fractional area change (FAC) was calculated using the following formula: \(((end-diastolic area) - (end-systolic area))/ (end-diastolic area)\). Also, regional systolic wall thickening was assessed in apical and mid-ventricular recordings. The local wall thickness was measured at end-diastole and systole in the infarct segments (anteroseptal and anterior wall) and one remote segment (inferolateral wall). Systolic wall thickening was subsequently determined by the following formula: \(((end-systolic wall thickness) - (end-diastolic wall thickness))/ (end-diastolic wall thickness)\).

Infarct volume and morphometry

After excision of the heart, the LV was isolated and cut into 5 slices from apex to base (online figure I). To discriminate infarct tissue from viable myocardium, the slices were incubated in 1% triphenyltetrazolium chloride (TTC, Sigma-Aldrich Chemicals, Zwijndrecht, Netherlands) in 37 °C Sörensen buffer (13.6 g/L KH2PO4+17.8 g/L Na2H PO4·2H2O, pH 7.4) for 15 min. All slices were scanned from both sides and in each slide the infarct area was compared to total area using digital planimetry software. After correction for the weight of the slices, infarct size was calculated as a percentage of the LV. Infarct thickness was depicted as the average of three measurements from endocardial to epicardial border per slice, whereas the border zone thickness was the average thickness of viable myocardium measured directly adjacent to both sides of the infarct.

Immunohistochemical staining

Collagen content, myocardial salvage index and cardiomyocyte size

Collagen content was assessed using Gomorri trichrome staining. In short, sections of the infarct and border zone, as well as sections of the remote area were deparaffinized and submerged in Bouin’s fixation solution (Sigma Aldrich, St. Louis, USA) at 56°C for 15 minutes. Nuclei were stained with haematoxylin, after which the slides were submerged in Trichrome-LG solution (Sigma Aldrich, St. Louis, USA). After treatment with 0.5% acetic acid solution for one minute, slides were mounted in Entallan (Merck, Darmstadt, Germany). Three random pictures were taken of each slide at 10x magnification, and collagen content was quantified using a customized software routine as mentioned before and depicted as percentage of the total surface area. The myocardial salvage index was calculated by dividing the area of viable myocardium in the infarct by the area that was composed of collagen.

Also, cardiomyocyte size was measured in trichrome-stained sections of border zone and remote myocardial segments. Three random pictures were taken at 40x magnification and the average surface area of at least 10 cardiomyocytes per field of view was determined. Surface area was only assessed of transversely cut cardiomyocytes in which a nucleus was visible to assure measuring the area at the mid level of the cardiomyocyte.

Capillary and arteriolar density
The blood vessel density was determined in border zone, remote area and the infarct area. In the border zone and remote area, blood vessel density was quantified by counting the amount of capillaries per mm². Blood vessel density in the infarct area however, was determined by quantifying arterioles, which was necessitated by the disarray of capillaries and pronounced aspecific staining. In brief, sections of the infarct border zone and remote myocardial segment were deparaffinized, rehydrated, pre-treated with trypsin EDTA (Lonza, Verviers, Belgium) and stained for isolectin-B₄ (Bandeiraea simplicifolia Isolectin-B₄ peroxidase, Sigma Aldrich, St. Louis, USA; (20 μg/ml)). Sections of the infarct area were stained for smooth muscle actin (SMA; clone 1A4, Sigma Aldrich, St. Louis, USA; 1:100). All sections were blocked in methanol/H₂O₂ solution for 30 minutes and incubated overnight at 4°C with isolectin-B₄ or SMA antibody solution. The slides for SMA staining were then washed and immersed in a secondary antibody (HRP-conjugated goat anti-mouse antibody, DAKO, Glostrup, Denmark) for 90 minutes. Subsequently, all slides were immersed in DAB solution (DAKO) for six minutes and finally mounted in Entellan. A technician blinded for the treatment allocation of the individual sheep took three random pictures of the border zone and remote myocardial segment or infarct area at 20x magnification after which capillaries and arterioles were quantified. Capillary density and arteriolar density were expressed as number per mm².

The micro-perfusion in the border zone and remote area was quantified as the number of capillaries per cardiomyocyte (capillary-to-cardiomyocyte ratio) and corrected for the collagen deposition in the extra-cellular matrix, and calculated using the following formula: \[ \left( \frac{\text{capillaries/mm}^2}{\text{cardiomyocytes/mm}^2} \right) \times (1 - \text{collagen content}) \].

**TUNEL, Ki67 and cKit staining**
Paraformaldehyde-fixed, paraffin-embedded heart sections of 5 μm thick were used for TUNEL, Ki-67, and c-Kit staining. The amount of apoptosis was quantified using a "In situ cell death detection kit" (Roche, Basel, Swiss) per the manufacturer’s instructions. Most antibodies have been used successfully in swine, but not sheep, by other laboratories. Antigen retrieval for Ki67 and cKit was done by boiling the slices for 30 minutes in 10mM citrate (pH 6). For Ki67 staining, sections were blocked in methanol/H₂O₂ solution for 30 minutes and incubated overnight at 4°C with Ki67 antibody (clone MIB-1, DAKO; 1:100) solution. The slides were then washed and immersed in a secondary antibody (HRP-conjugated goat anti-mouse antibody, DAKO) for 90 minutes, after which they were immersed in DAB solution (DAKO). Pictures of multiple fields (400x) were used to quantify the frequency of Ki67 staining. cKit staining was performed by immersing the slides in cKit antibody solution (ab5506, Abcam, Cambridge, UK; 1:100) together with anti-cTnI (mouse monoclonal antibody clone 8I-7, Spectral diagnosis, 1:100) to detect myocyte filaments. Samples were posttreated with fluoroisothiocyanate (FITC) conjugated anti-rabbit and TRITC conjugated anti-mouse antibody (Dako). Nuclei were stained with DAPI (Vectashield). Multiple fields were photographed using an Olympus IX55 fluorescence microscope, after which cKit+ cells were quantified.
Results

Nuclear cell tracking experiments
In two separate sheep, bio-distribution was assessed following intracoronary infusion of 37.5 million Indium$^{111}$ labeled MPC. The Indium$^{111}$ labeling efficiency was 79.5 ± 7.5%, and cell viability exceeded 85% after the labelling procedure. *Ex vivo* quantification of Indium$^{111}$ uptake in all major organs estimated a cell uptake in the heart of 40.8% of the total cell dose in sheep 1 and 53.5% in sheep 2, whereas lungs (13.7% and 6.9%), kidneys (2.3% and 1.7%), liver (5.6% and 2.6%), spleen (0.8% and 0.3%), and pericardium (0.3% and 0.1%) had limited uptake (online figure IIA). The residual activity was predominantly detected in the infusion and urinary catheter systems. In the heart, the MPC were retained in the perfusion territory of the LAD (online figure IIB/C), *i.e.* the anterior and anteroseptal wall. No activity was detected in remote myocardial segments (online figure IIB).

Measurement of cell diameter of mesenchymal precursor cells
Ovine MPC have a median diameter of 13 micron and a mean diameter of 13.2 ± 2.2 micron (online figure III)
**Discussion**

*Analysis of cardiac function*

In this study, cardiac function and volumes were assessed using both PV-loop analysis and echocardiography. Invasive hemodynamics by analysis of the pressure-volume relation analysis renders reliable and reproducible quantification of the LV volumes, and thus LVEF, throughout the cardiac cycle.\(^{12,13}\) Also, indices of intrinsic myocardial contractile function can be determined that are independent of pre- and afterload conditions, which are known to differ substantially between the acute phase of the AMI and at eight weeks follow up.\(^{8,14}\) Echocardiography analysis showed comparable pre-AMI conditions between groups, provided supportive data on global and regional cardiac function, and corroborated PV-loop data. Importantly, PV-loop and echocardiography data were acquired and analyzed by separate technicians, who were blinded for the treatment allocation of the individual sheep.
References
Online figure I.

Post-mortem preparation of the heart and TTC staining. After excision, the hearts were cut into five slices in a bread-loaf manner, after which the slices were stained by TTC. TTC staining turns viable myocardium red, whereas infarcted tissue remains white. This facilitates distinction between infarct area, border zones and remote myocardial segments. The slice marked with * represents the apical segment that was used for analysis, the slice marked with † represents the mid ventricular slice.
Online figure II.

**Biodistribution of MPC as assessed by Indium\textsuperscript{111} labelling.** IIA. *Ex vivo* scanning of all major organs two hours following intracoronary infusion of 37.5 million MPC. IIB. Top picture: bread-loafed slices from apex to base; middle picture: MPC distribution in these slices; bottom picture: overlay. MPC were only targeted to the perfusion territory of the culprit artery (arrows), whereas no signs of activity were found in remote myocardial segments. IIC. Left picture: anterior view of the intact heart; middle picture: MPC distribution in the anterior wall and apex; right picture: overlay. Arrows: left anterior descending artery; MPC: mesenchymal precursor cells.
Online figure III.

Light photomicrographs of mesenchymal precursor cells at 1000x magnification.
**Online table I.** Coronary flow, ventricular arrhythmias and death

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Treatment group</th>
<th>p-value</th>
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<tbody>
<tr>
<td><strong>Coronary flow</strong></td>
<td></td>
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</tr>
<tr>
<td>Reduction of TIMI* flow</td>
<td>0/12</td>
<td>0/22</td>
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<tr>
<td><strong>Death</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventricular fibrillation</td>
<td>2/12</td>
<td>0/22</td>
<td>0.144</td>
</tr>
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<td>0.543</td>
</tr>
<tr>
<td>All</td>
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<td>2/22</td>
<td>0.612</td>
</tr>
<tr>
<td><strong>Ventricular arrhythmia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventricular fibrillation</td>
<td>2/12</td>
<td>0/22</td>
<td>0.144</td>
</tr>
<tr>
<td>Ventricular tachycardia</td>
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<td>0/22</td>
<td>0.371</td>
</tr>
<tr>
<td>All</td>
<td>3/12</td>
<td>0/22</td>
<td>0.059</td>
</tr>
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</table>

*TIMI: thrombolysis in myocardial infarction

P-values were determined using a two-sided Fischer’s exact test.
Online table II. Pressure–volume loop derived parameters.

<table>
<thead>
<tr>
<th>reference</th>
<th>control</th>
<th>MPC</th>
<th>control</th>
<th>MPC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=12</td>
<td>n=10</td>
<td>n=20</td>
<td>n=10</td>
<td>n=20</td>
<td></td>
</tr>
</tbody>
</table>

**Volumetric parameters**

- **HF (beats/min)**
  - Pre AMI: 70.1 ± 3.4, Post AMI: 84.8 ± 4.0, Post AMI: 76.5 ± 5.2, 8 weeks: 92.6 ± 10.0, 8 weeks: 69.9 ± 4.4, P = 0.11

- **Volumes**
  - End systolic volume (mL): 56.1 ± 3.3, 73.5 ± 2.8, 71.1 ± 1.2, 102.8 ± 4.0, 68.3 ± 1.8, P < 0.001
  - End diastolic volume (mL): 138 ± 7.8, 135 ± 2.0, 137 ± 1.8, 178 ± 8.0, 149 ± 4.1, P < 0.001

**Systolic function**

- **Ejection fraction (%)**
  - Pre AMI: 63.5 ± 1.7, Post AMI: 44.2 ± 1.5, Post AMI: 44.7 ± 1.0, 8 weeks: 40.7 ± 2.6, 8 weeks: 52.8 ± 0.7, P < 0.001

- **Elastance (E<sub>es</sub>)**
  - Pre AMI: 1.59 ± 0.12, 0.96 ± 0.07, Post AMI: 0.99 ± 0.06, 0.89 ± 0.05, 0.126 ± 0.1, 0.003

- **PRSW<sup>‡</sup> (mmHg)**
  - Pre AMI: 47.7 ± 2.8, 27.4 ± 1.0, Post AMI: 28.5 ± 1.6, 33.5 ± 1.44, 41.6 ± 1.9, 0.008

- **End systolic pressure (mmHg)**
  - Pre AMI: 86.8 ± 4.5, 70.5 ± 6.0, Post AMI: 69.8 ± 3.8, 90.5 ± 6.6, 84.4 ± 3.7, 0.51

- **Stroke volume (mL)**
  - Pre AMI: 80.2 ± 5.7, 65.9 ± 2.4, Post AMI: 72.0 ± 3.0, 71.4 ± 6.2, 78.1 ± 1.9, 0.41

- **Stroke work (mL.mmHg)**
  - Pre AMI: 6511 ± 484, 3707 ± 179, Post AMI: 3954 ± 232, 5970 ± 375, 6331 ± 325, 0.47

- **Cardiac output (L/min)**
  - Pre AMI: 5.5 ± 0.3, 4.8 ± 0.24, Post AMI: 4.5 ± 0.3, 6.2 ± 0.5, 5.4 ± 0.3, 0.22

- **dP/dtmax**
  - Pre AMI: 1136 ± 90, 980 ± 38, Post AMI: 1027 ± 71, 1091 ± 82, 1155 ± 75, 0.15

- **dP/dTmax / EDV**
  - Pre AMI: 9.1 ± 0.7, 7.3 ± 0.2, Post AMI: 7.6 ± 0.6, 7.5 ± 0.7, 8.5 ± 0.4, 0.042

- **tPER<sup>§</sup> (msec)**
  - Pre AMI: 165 ± 11, 165 ± 12, Post AMI: 147 ± 9, 163 ± 22, 165 ± 9, 0.30

**Diastolic function**

- **Stiffness**
  - Pre AMI: 0.05 ± 0.01, 0.09 ± 0.03, Post AMI: 0.06 ± 0.01, 0.04 ± 0.01, 0.04 ± 0.00, 0.22

- **V<sub>0</sub> (mL)**
  - Pre AMI: 75.4 ± 4.1, 71.0 ± 3.8, Post AMI: 76.1 ± 1.1, 99.8 ± 4.7, 83.9 ± 2.1, 0.001

- **V<sub>30</sub> (mL)**
  - Pre AMI: 167 ± 16, 185 ± 15, Post AMI: 185 ± 7, 241 ± 24, 213 ± 8, 0.047

- **Tau**
  - Pre AMI: 29.5 ± 1.5, 37.7 ± 7.3, Post AMI: 32.9 ± 2.1, 27.0 ± 1.8, 28.2 ± 0.9, 0.27

- **End diastolic pressure (mmHg)**
  - Pre AMI: 8.2 ± 1.5, 11.0 ± 4.7, Post AMI: 7.6 ± 0.9, 9.7 ± 1.7, 6.0 ± 1.0, 0.08

- **dP/dtmin**
  - Pre AMI: -1102 ± 77, -818 ± 56, Post AMI: -823 ± 67, -1042 ± 114, -1126 ± 51, 0.29

- **dP/dtmin / ESV**
  - Pre AMI: -12.5 ± 0.9, -11.1 ± 0.6, Post AMI: -11.2 ± 0.7, 11.1 ± 1.0, 15.3 ± 0.9, 0.008

- **tPFR<sup>‖</sup> (msec)**
  - Pre AMI: 623 ± 42, 525 ± 30, Post AMI: 604 ± 36, 505 ± 30, 648 ± 52, 0.023
*mesenchymal precursor cells; †acute myocardial infarction; ‡preload recruitable stroke work; §top peak ejection rate; ¶top peak filling rate
### Online table III. Echocardiographic volumes and ejection fraction

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MPC-treated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEF BL (%)</td>
<td>51.3 ± 2.5</td>
<td>52.2 ± 2.5</td>
<td>0.77</td>
</tr>
<tr>
<td>LVEF post (%)</td>
<td>43.1 ± 1.2</td>
<td>42.4 ± 1.4</td>
<td>0.58</td>
</tr>
<tr>
<td>LVEF 4WFU (%)</td>
<td>42.0 ± 1.7</td>
<td>46.3 ± 0.9</td>
<td>0.009</td>
</tr>
<tr>
<td>LVEF 8WFU (%)</td>
<td>37.3 ± 1.9</td>
<td>47.7 ± 1.2</td>
<td>0.001</td>
</tr>
<tr>
<td>ESV BL (mL)</td>
<td>58.5 ± 4.4</td>
<td>54.9 ± 3.3</td>
<td>0.51</td>
</tr>
<tr>
<td>ESV post (mL)</td>
<td>67.8 ± 3.7</td>
<td>62.8 ± 4.2</td>
<td>0.33</td>
</tr>
<tr>
<td>ESV 4WFU (mL)</td>
<td>90.4 ± 7.8</td>
<td>65.9 ± 4.8</td>
<td>0.025</td>
</tr>
<tr>
<td>ESV 8WFU (mL)</td>
<td>115.8 ± 16.5</td>
<td>71.3 ± 6.9</td>
<td>0.042</td>
</tr>
<tr>
<td>EDV BL (mL)</td>
<td>119.7 ± 6.7</td>
<td>115.1 ± 5.6</td>
<td>0.58</td>
</tr>
<tr>
<td>EDV post (mL)</td>
<td>119.3 ± 7.2</td>
<td>112.6 ± 5.9</td>
<td>0.39</td>
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<tr>
<td>EDV 4WFU (mL)</td>
<td>155.2 ± 10.6</td>
<td>123.0 ± 9.2</td>
<td>0.018</td>
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<tr>
<td>EDV 8WFU (mL)</td>
<td>182.9 ± 22.5</td>
<td>136.2 ± 12.0</td>
<td>0.037</td>
</tr>
</tbody>
</table>

BL: baseline; post: directly post myocardial infarction; LVEF: left ventricular ejection fraction; 4WFU/8WFU: 4 and 8 week follow up; ESV: end systolic volume; EDV: end diastolic function.