Effects of Intracoronary Infusion of Escalating Doses of Cardiac Stem Cells in Rats With Acute Myocardial Infarction

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Background—Although c-kit<sup>+</sup> cardiac stem cells (CSCs) preserve left ventricular (LV) function and structure after myocardial infarction, CSC doses have been chosen arbitrarily, and the dose–effect relationship is unknown.

Methods and Results—Rats underwent a 90-minute coronary occlusion followed by 35 days of reperfusion. Vehicle or CSCs at 5 escalating doses (0.3×10<sup>6</sup>, 0.75×10<sup>6</sup>, 1.5×10<sup>6</sup>, 3.0×10<sup>6</sup>, and 6.0×10<sup>6</sup> cells/heart) were given intracoronarily 4 h after reperfusion. The lowest dose (0.3×10<sup>6</sup>) had no effect on LV function and morphology, whereas 0.75, 1.5, and 3.0×10<sup>6</sup> significantly improved regional and global LV function (echocardiography and hemodynamic studies). These 3 doses had similar effects on echocardiographic parameters (infarct wall thickening fraction, LV end-systolic and end-diastolic volumes, LV ejection fraction) and hemodynamic variables (LV end-diastolic pressure, LV dP/dt<sub>max</sub>, preload adjusted maximal power, end-systolic elastance, preload recruitable stroke work) and produced similar reductions in apoptosis, scar size, infarct wall thinning, and LV expansion index and similar increases in viable myocardium in the risk region (morphometry). Infusion of 6.0×10<sup>6</sup> CSCs markedly increased postprocedural mortality. Green fluorescent protein and 5-bromo-2′-deoxyuridine staining indicated that persistence of donor cells and formation of new myocytes were negligible with all doses.

Conclusions—Surprisingly, in this rat model of acute myocardial infarction, the dose–response relationship for intracoronary CSCs is flat. A minimal dose between 0.3 and 0.75×10<sup>6</sup> is necessary for efficacy; above this threshold, a 4-fold increase in cell number does not produce greater improvement in LV function or structure. Further increases in cell dose are harmful.

Key Words: left ventricular function ■ myocardial infarction ■ myocardial ischemia ■ myocardial regeneration ■ progenitor cells

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However, despite these encouraging results, many questions pertaining to the therapeutic efficacy of CSCs remain unanswered. One of the most important is the dose of CSCs, which to date has been chosen rather arbitrarily; specifically, what is the dose–response relationship for CSC therapy? In our previous studies, we found that intracoronary administration of 1×10<sup>6</sup> CSCs produced beneficial effects on LV function designed to investigate the safety and feasibility of autologous CSC infusion in patients with severe heart failure resulting from ischemic cardiomyopathy. The potential therapeutic utility of c-kit<sup>+</sup> CSCs is further supported by the fact that these cells can be isolated from small fragments of cardiac tissue and expanded for subsequent autologous administration.

Received March 21, 2015; accepted May 15, 2015.
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Circ Heart Fail is available at http://circheartfailure.ahajournals.org
DOI: 10.1161/CIRCHEARTFAILURE.115.002210
and structure in rats after acute and chronic MI. However, the optimal cell dosage for this approach, and whether higher doses would result in greater benefit, remains unknown; as mentioned above, the 1x10^6 dose was chosen arbitrarily. In the current study, we used the same model as in our previous investigation (intracoronary administration of CSCs in rats with acute MI) and infused escalating cell doses to delineate the dose-dependency of the effects of CSCs on cardiac repair. Surprisingly, we found that increasing the dose of CSCs above the therapeutic threshold does not result in greater benefit.

Methods
All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Eighth Edition, Revised 2010) and with the guidelines of the Animal Care and Use Committee of the University of Louisville, School of Medicine (Louisville, KY).

Isolation and Expansion of c-kit CSCs
C-kit CSCs were prepared using a modification of the method described by Beltrami et al. Briefly, cardiac cells were isolated from adult male Fischer 344 rats (4–6 months of age), the nonmyocyte population was separated by myocyte by gravity sedimentation, counted (number of cells determined), and centrifuged at 600g for 10 minutes at room temperature. Small intact cells were resuspended and the cells seeded onto two 150-mm dishes in rat cardiac stem cell media (P12K medium with 5% FBS [HyClone]), 10 ng/mL basic fibroblast growth factor (Peprotech), and 10 ng/mL leukemia inhibitory factor (Chemicon). After 6 days of expansion, c-kit cells were sorted with magnetic beads (Dynal) coated with c-kit antibody (H-300, Santa Cruz). c-kit cells were collected with magnetic beads (Dynal) coated with anti-rabbit IgG and maintained in culture with rat CSC medium. The recovery of c-kit CSCs was determined by flow cytometry staining with an r-phycocerythrin-conjugated rat monoclonal anti-c-kit antibody (Chemicon). CSCs were labeled with green fluorescent protein with an r-phycoerythrin-conjugated rat monoclonal anti-c-kit antibody. c-kit CSCs from passages 4 to 6 were used in all in vitro studies of post-MI CSC transplantation. The purity of sorted cells was determined by fluorescence-activated cell sorting.

Experimental MI and Cell Injection
MI and intracoronary transfusion procedures were performed as previously. Briefly, Female Fischer 344 rats (age 10–12 weeks; weight 174±8 g) were anesthetized with ketamine (37 mg/kg) and xylazine (5 mg/kg) and mechanically ventilated. Anesthesia was maintained with isoflurane (1% to 3%). All animals underwent a 90-minute occlusion of the left anterior descending coronary artery followed by reperfusion (Figure 1), and the chest was closed. Four hours after reperfusion, rats were reanesthetized, the chest reopened, and a thin catheter (Intracath, 22G, Becton Dickinson) was advanced into the aortic root via the LV apex. Washed CSCs at doses of 0 (vehicle), 0.3, 0.75, 1.5, 3.0, or 6.0 million (x10^6) were suspended in 1 mL PBS and injected into the aortic root (Figure 1). Rats were followed up for 35 days after cell application and then euthanized for histological studies. For tracking proliferating cells, rats were fed 5-bromo-2′-deoxyuridine (BrdU) in drinking water during the 35-day follow-up (Figure 1).

Transthoracic Echocardiography and Hemodynamic Measurements
Echocardiography was performed under light anesthesia (pentobarbital, 25 mg/kg, IP) as described previously using an HDI 5000 echocardiography machine (Philips Medical Systems, Best, The Netherlands) equipped with 15–7 MHz linear broadband and 12–5 MHz phased array transducers. Serial echocardiograms were obtained at baseline (2 days before coronary occlusion) and at 48 h and 35 days after treatment. Short-axis 2D and M mode images were recorded. Systolic and diastolic anatomic parameters were obtained from M-mode tracings at the midapical level. LV volume was calculated by Teichholz formula and the ejection fraction was calculated by M-mode quantification formula. LV area was measured from short-axis 2D images. All measurements were averaged in 3 consecutive cardiac cycles and analyzed off-line by a single blinded observer using the COMPACS image analysis software.

Hemodynamic measurements were performed at the 35-day follow-up, just before euthanization, using a 2.0 F Millar’s Micro-Tip® ultraminiature PV loop catheters (SPR-869; Millar Instruments, Houston, TX) as described. In brief, rats were anesthetized with ketamine (37 mg/kg) and xylazine (5 mg/kg), intubated, and mechanically ventilated. Anesthesia was maintained with 1% isoflurane and the core temperature kept at 37.0°C with a heating pad throughout the study. A 2F PV loop catheter (SPR-869, Millar Instruments) was inserted into the right carotid artery and advanced into the LV cavity. The right jugular vein was cannulated for fluid administration. After 20 minutes of stabilization, the PV signals were recorded continuously with an ARIA PV conductance system (Millar Instruments) coupled with a Powerlab/4SP A/D converter (AD Instruments), stored, and displayed on a personal computer. PV relations were assessed by transiently compressing the inferior vena cava with a cotton swab. Parallel conductance from surrounding structures was calculated by injecting a small bolus of 30% NaCl through the jugular venous cannula. LV end-diastolic pressure, dP/dt, preload adjusted maximal power, end-systolic elastance, and preload recruitable stroke work were calculated using the PVAN software program (Millar).

Morphometry and Histology
After the hemodynamic measurements, a polyelectrolyte catheter filled with phosphate buffer (0.2 mol/L, pH 7.4) and heparin (100 IU/mL) was advanced to the ascending aorta via the right carotid artery. In rapid succession, the heart was arrested in diastole by injecting 1.0 mL of a mixture of cadmium chloride (100 mmol/L), potassium chloride (3 mol/L) through the jugular venous cannula. The heart was then excised and perfused retrogradely with phosphate buffer for 3 minutes to flush out residual blood in the coronary circulation, followed by perfusion with 10% neutral buffered formalin solution for fixation.
15 minutes. Perfusion pressure was maintained between 60 and 80 mmHg, whereas end-diastolic pressure was kept at 8 mmHg. After perfusion-fixation, the atria and right ventricle were dissected from the left ventricle. The LV weight, volume, and the longitudinal intracavitary axis (LV cavity length) were measured to evaluate LV gross morphology. The heart was cut into 4 transverse slices (2-3 mm thick), which were processed, embedded in paraffin, sectioned at 4-μm intervals, and stained with Masson’s trichrome, picrosirius red, or antibodies against specific cell markers. Images were acquired digitally and analyzed using NIH ImageJ (1.44p). From the Masson’s trichrome-stained images, morphometric parameters, including risk area, infarct size, and viable myocardium in the risk, were measured in each section. To evaluate cardiac fibrosis, LV sections were stained with picrosirius red, and collagen content was quantitated in images taken under polarized light.

### Immunohistochemistry

Immunofluorescence staining was performed in formalin-fixed, 4-μm-thick histological sections and analyzed by confocal microscopy for GFP, BrdU, α-sarcosomic actin (α-sarc), TUNEL-positive nuclei, and isolecitin. Engraftment and proliferation in LV cross-sections were assessed by staining with antibodies against GFP or BrdU, respectively, which were then counterstained with α-sarc. Capillary density was determined in sections stained with FITC-conjugated isolecitin. Apoptotic cells were identified by TUNEL staining. Images were acquired, and cells staining positive for each of these markers counted and normalized per mm².

### Statistical Analysis

All data are expressed as means±SEM. Echocardiographic data were performed by 2-way repeated-measures ANOVA followed by Student’s t-tests with Bonferroni correction for intra- and intergroup comparisons. All parametric data, including morphometric, histological, immunohistochemical, and hemodynamic data, were analyzed by 1-way ANOVA followed by the Student’s t-tests with Bonferroni correction for intergroup comparisons. Mortality was analyzed by the χ² test. All analyses were conducted with SigmaStat3.5. Values of P<0.05 were considered significant.

### Results

#### Exclusions and Gross Measurements

Of the 82 rats enrolled into this study, 32 died during the course of the experiment (Table). One (in the 0.75×10⁶ group) of the 32 rats died of ventricular fibrillation during coronary occlusion and the remaining 31 within 48 h after the intra-coronary infusion. The mortality rate was similar among the groups (28.6% with vehicle, 30.0% with 0.3×10⁶, 26.7% with 0.75×10⁶, 30.8% with 1.5×10⁶, and 33.3% with 3.0×10⁶), except for the 6.0×10⁶ group, in which mortality was 80% (12 out of 15), significantly higher (P<0.05) than in the other 5 groups (Table). Thus, a total of 50 rats (10 in the vehicle, 7 in the 0.3×10⁶, 11 in the 0.75×10⁶, 9 in the 1.5×10⁶, 10 in the 3.0×10⁶, and 3 in the 6.0×10⁶ group) completed the protocol. Because only 3 rats survived in the 6.0×10⁶ group and because of the obvious toxicity of this CSC dose, these animals were not included in the analysis of cardiac function and structure.

LV weight and LV/body weight ratio were lower in rats treated with CSCs at doses of 0.75×10⁶ or higher compared with the vehicle group. Postmortem gross measurements conducted in the diastolically arrested, perfusion-fixed heart revealed a shorter LV longitudinal axis and a smaller chamber volume in rats receiving 0.75 to 3.0×10⁶ CSCs compared with rats receiving vehicle (Figure 2).

#### Myocardial Engraftment of Transplanted CSCs

Myocardial engraftment of the transplanted CSCs (or their progeny) was determined by counting the number of GFP⁺ cells on LV sections after immunofluorescence staining for GFP (Figure 3A). As expected, no GFP signal was detected in the 10 hearts in the vehicle-treated group. GFP⁺ cells in the 0.75, 1.5, and 3.0×10⁶ groups were found in 9/11, 8/9, and 9/10 hearts, respectively, in the risk region and in 5/11, 6/9, and 5/10 hearts, respectively, in the remote region. Quantitative analysis of the hearts with detectable GFP signals revealed that the number of GFP⁺ cells increased dose-dependently with the escalating doses both in the risk and remote (noninfarcted) regions (Figure 3B). At any given dose, the number of GFP⁺ cells was greater in the risk region versus the remote region; however, in both cases, the absolute number of GFP⁺ cells was extremely low (<50 cells/10000 nuclei in the risk region and <20 cells/10000 nuclei in the remote region; Figure 3B). Thus, intracoronary infusion of CSCs resulted in a dose-dependent increase in myocardial engraftment at 35 days after transplantation of CSCs, but the number of engrafted cells was minuscule, too low to account for functional or structural benefits.

#### LV Morphometric Analysis

Morphometric analysis indicated that neither infarct size nor viable myocardium was altered in the 0.3×10⁶ group compared with vehicle (Figure 4). Despite similar risk regions among groups, CSCs at doses of 0.75×10⁶ and higher significantly reduced infarct size (54.4%±2.1% in the risk region in the vehicle group versus 45.1%±2.0% in the 0.75×10⁶, 44.2%±2.3% in the 1.5×10⁶, and 43.8%±3.7% in the 3.0×10⁶, P<0.05 for all comparisons), increased infarcted wall thickness (1.44±0.07 mm in the vehicle group versus 2.03±0.12 in the 0.75×10⁶ [P<0.01], 1.98±0.16 in the 1.5×10⁶ [P<0.05], and 2.21±0.14 in the 3.0×10⁶ [P<0.01]), increased the amount of viable tissue within the risk region (45.6%±2.1% in the vehicle group versus 54.9%±2.0% in the 0.75×10⁶, 55.8%±2.3% in the 1.5×10⁶, and 56.2%±3.7% in the 3.0×10⁶, P<0.05 for all comparisons), and limited LV dilation, as indicated by the LV expansion index (0.83±0.04 in the vehicle group versus 0.56±0.04 in the 0.75×10⁶, 0.58±0.05 in the 1.5×10⁶, and 0.54±0.05 in the 3.0×10⁶, P<0.01 for all comparisons; Figure 4). Thus, in terms of morphometric variables, the

### Table. Enrollment and Exclusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Animals Initial Enrollment</th>
<th>Animals Completed Protocol</th>
<th>Mortality, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>14</td>
<td>10</td>
<td>28.6*</td>
</tr>
<tr>
<td>0.3×10⁶ CSCs</td>
<td>10</td>
<td>7</td>
<td>30.0*</td>
</tr>
<tr>
<td>0.75×10⁶ CSCs</td>
<td>15</td>
<td>11</td>
<td>26.7*</td>
</tr>
<tr>
<td>1.5×10⁶ CSCs</td>
<td>13</td>
<td>9</td>
<td>30.8*</td>
</tr>
<tr>
<td>3.0×10⁶ CSCs</td>
<td>15</td>
<td>10</td>
<td>33.3*</td>
</tr>
<tr>
<td>6.0×10⁶ CSCs</td>
<td>15</td>
<td>3</td>
<td>80.0</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>50</td>
<td>39.0</td>
</tr>
</tbody>
</table>

CSC indicates cardiac stem cells.

*P<0.05 vs 6.0×10⁶ CSCs.
threshold for CSC efficacy lies between the 0.3 and 0.75×10^6 doses; above this threshold, there was no indication of a dose-dependent effect of CSCs as the dose increased from 0.75 to 3.0×10^6 (Figure 4).

Collagen content in the risk region was reduced by CSC administration at a dose of 0.75×10^6 or higher (P<0.05 versus vehicle for all groups), but not at the dose of 0.3×10^6; again, the reduction did not exhibit a dose-dependent pattern (Figure 5). None of the CSC doses had any effect on collagen in the remote region (Figure I in the Data Supplement).

**Echocardiographic and Hemodynamic Analyses**

At baseline (before MI), all parameters of LV function, measured by echocardiography, were similar among groups (Figure 5). At 48 h after MI, the degree of LV dysfunction did not differ among the groups, indicating that the extent of injury sustained during MI was comparable. As expected, in vehicle-treated rats, the infarct wall thickness in systole, infarct wall thickening fraction, fractional shortening, and ejection fraction decreased, whereas LV end-systolic diameter, LV end-systolic area, and LV end-systolic and end-diastolic volumes (LVEDV) increased at the 35-day follow-up compared with baseline (Figure 5). In rats that received 0.3×10^6 CSCs, none of these parameters was different from the vehicle group, indicating that this dose is not effective. In contrast, in rats that received a dose of 0.75×10^6 CSCs or greater, these parameters were improved, so that the infarct wall thickening fraction, fractional shortening, ejection fraction, and fractional area change were greater and the LV end-systolic diameter, LV end-systolic area, LV end-systolic and LV end-diastolic volumes were smaller compared with vehicle-treated rats (Figure 5B). However, in the groups receiving 0.75, 1.5, and 3.0×10^6, there was no discernible dose-dependent pattern with respect to any of these echocardiographic variables at the 35-day follow-up (Figure 5B).

Taken together, the echocardiographic data indicate that CSCs improved LV function when the dose exceeded a threshold between 0.3 and 0.75×10^6; increasing the cell dose above 0.75×10^6 did not produce greater effects.

Consistent with the echocardiographic data, hemodynamic studies (performed before euthanasia using the conductance [Millar] catheter) showed that the lowest dose of CSCs (0.3×10^6) did not alter hemodynamic variables compared with the vehicle-treated group (Figure 6). However, CSCs at doses of 0.75×10^6 and greater produced significant improvements in LV end-diastolic pressure, dP/dt max, dP/dt max/EDV, preload adjusted maximal power, end-systolic elastance, and preload recruitable stroke work (Figure 6). As was the case for the echocardiographic data, there was no appreciable difference between doses of 0.75, 1.5, and 3.0×10^6.

**Cellular Apoptosis**

The number of TUNEL-positive cells was counted in ≈0.5% of total nuclei in both the risk and remote regions (Figure 7). The 0.75×10^6 dose produced a dramatic reduction in apoptotic cells in both regions compared with vehicle; the effects of the 1.5×10^6 and 3.0×10^6 doses were similar (Figure 7).

**Cellular Proliferation**

BrdU incorporation was analyzed in the vehicle, 0.75×10^6, 1.5×10^6, and 3.0×10^6 groups, in which rats received BrdU in the drinking water during the 35-day follow-up. The total number of BrdU-positive (BrdU^pos) cells was higher in the 3.0×10^6 group than in vehicle-treated hearts, both in the risk region and in the remote region (Figure 8B, upper panel). Although the total number of BrdU^pos cells was higher in the
0.75 and 1.5×10^6 groups than in the vehicle group, the differences were not statistically significant.

Co-staining of tissue with BrdU and α-sarc antibodies showed no BrdU^pos/α-sarc^pos cells in the vehicle-treated hearts (Figure 8B, middle panel). A dose-dependent increase was noted in the 3 CSC-treated groups, both in the risk and remote regions (Figure 8B, middle panel). There were no significant differences among groups in either the risk or the remote region. Further analyses after co-staining with GFP revealed that a minority of the BrdU^pos/α-sarc^pos cells expressed GFP, indicating that they were the transplanted cells or their progeny (Figure 8B, lower panel).

Taken together, the BrdU data indicate that administration of escalating doses of CSCs was associated with a dose-dependent increase in the numbers of BrdU^pos and BrdU^pos/α-sarc^pos cells, suggesting that CSC treatment dose-dependently promoted myocyte proliferation. A minority of newly formed myocytes were derived from the transplanted CSCs. However, because the absolute numbers of BrdU^pos/α-sarc^pos cells were extremely low (<10/10^4 nuclei), it is unlikely that this increase in myocyte proliferation contributed importantly to the beneficial effects of CSCs.

**Capillary Density**

Isolectin-stained capillaries averaged ≈800/mm^2 both in the risk and remote regions of the vehicle-treated hearts (Figure II in the Data Supplement). Three escalating doses of CSCs (0.75, 1.5, and 3.0×10^6) dose-dependently increased capillary...
density in both the risk and remote regions; compared with vehicle-treated hearts, the density was significantly higher in the $1.5 \times 10^6$ and $3.0 \times 10^6$ groups in the risk region and in the $3.0 \times 10^6$ group in the remote region (Figure II in the Data Supplement).

**Discussion**

The present study was undertaken to determine whether the effects of CSC therapy follow a dose-dependent pattern. The salient results can be summarized as follows. In rats undergoing intracoronary infusion of syngeneic CSCs after acute MI, (i) $0.3 \times 10^6$ CSCs did not produce any beneficial effect on LV structure or function 35 days later; (ii) escalating doses of CSCs ($0.75 \times 10^6$, $1.5 \times 10^6$, and $3.0 \times 10^6$) resulted in a dose-dependent increase in the myocardial engraftment of the transplanted cells at 35 days, but the absolute number of these cells was so low (<50 cells/10000 nuclei) that it was unlikely to contribute significantly to the beneficial effects of CSC therapy; (iii) $6.0 \times 10^6$ CSCs resulted in significantly higher mortality, possibly because of extensive microembolization; (iv) doses of $0.75$, $1.5$, and $3.0 \times 10^6$ resulted in improved LV structure, as manifested by a shortened LV longitudinal axis, decreased chamber volume, reduced scar size, thicker infarcted LV wall, restrained LV dilation, reduced fibrosis, and increased content of viable myocardium in the risk region; (v) this improvement in LV structure was associated with improved regional and global LV function, as demonstrated by 2 independent techniques: echocardiography (increased infarct wall thickening fraction, reduced LV end-systolic volume, and increased LVEF) and hemodynamic studies (lowered LV end-diastolic pressure enhanced $dP/dt_{max}$ and increased preload adjusted maximal power, end-systolic elastance, and $dP/dt_{max}/EDV$); (vi) neither the improvement in structure nor the improvement in function associated with $0.75 \times 10^6$, $1.5 \times 10^6$, and $3.0 \times 10^6$ CSCs showed a dose-dependent pattern; (vii) all 3 doses of CSCs reduced apoptosis to a similar extent in both the infarcted and the noninjured myocardium; and (viii) these 3 doses produced a dose-dependent increase in the formation of new myocytes and capillaries, but the number of new myocytes was exceedingly low. Taken together, these results lead to the conclusion that, in the rat, the benefits of intracoronary infusion of CSCs after acute MI are not dose-dependent. Although many previous studies have examined the effects of CSCs in rodents, to our knowledge, this is the first analysis of the dose-dependence of these effects.

The reason for using the rat MI model and this range of CSC doses was that our previous studies demonstrated that intracoronary administration of $1 \times 10^6$ CSCs produced beneficial effects on LV function and morphology in this same rat model. We used intracoronary infusion of CSCs, rather than intramyocardial injection, for several reasons. Clinically, the technique for intracoronary delivery is similar to that used for coronary angioplasty and allows rapid translation because...
many interventional cardiologists are well versed with this method. In addition, it is the most popular mode of cell delivery in the clinical setting, especially after acute MI, because it can be done simultaneously during a percutaneous coronary intervention for treating stenotic coronary arteries. It also enables stem cells to be selectively infused into the target area. Finally, intracoronary administration allows the cells to home to and engraft within the areas bordering the infarct zone in a broader, more homogeneous manner.

We found that the lowest dose of CSCs (0.3×10^6) was ineffective, whereas the 3 higher doses of 0.75, 1.5, and 3.0×10^6 exerted clear beneficial effects on LV function (Figures 5 and 6) and structure (Figures 2, 4 and 5), which, however, were not related to the dose. Thus, the threshold for efficacy in this model is between 0.3 and 0.75×10^6; above this threshold, the benefits do not increase as the dose of CSCs increases 4-fold (from 0.75 to 3.0×10^6). Our echocardiographic results were corroborated by the hemodynamic studies, which provide an assessment of function that is completely independent of echocardiography and that, unlike the echocardiographic data, is based on load-independent variables, that is, dP/dt max/EDV, preload adjusted maximal power, end-systolic elastance, and preload recruitable stroke work.

In contrast to the functional data, myocyte and CSC proliferation and capillary density exhibited a dose-related pattern with doses of 0.75, 1.5, and 3.0×10^6 (Figure 8; Figure II in the Data Supplement). However, the increase in dose did not produce a proportionate increase in myocyte and CSC proliferation. Furthermore, the absolute cell numbers were extremely small and unlikely to have functional significance: for example, the number of BrdU^{pos}/α-sarc-pos cells was <10/10000 nuclei, and the number of BrdU^{pos}/α-sarc-pos/GFP^{pos} cells was <4/10000 nuclei (Figure 8; Figure II in the Data Supplement).

The effects of the highest dose of CSCs (6.0×10^6) were not analyzed for 2 main reasons: first, the group size was too small (n=3) for meaningful statistical analysis, and second, the mortality in this group was much higher than in the other 4 groups (80% versus 27% to 33%, P<0.05 for all groups), making it possible that only the healthiest animals (those with either less cardiac injury or better overall conditions) may have survived the CSC infusion, thereby skewing the outcome in a manner that is unrelated to CSCs. Clearly, the maximal dose of CSCs that should be considered for experimental studies is the highest dose that does not cause safety concerns.

Most previous investigators studying the effects of different doses of cells with a regenerative capacity have found a positive dose–efficacy relationship. For instance, intramyocardial injection of escalating doses of human bone marrow–derived CD34^{pos} cells in rats, human undifferentiated mesenchymal stem cells and differentiated cardiomyocyte-like cells in rats, and human cardiospheres in mice has been reported to impart dose-dependent functional and histological benefits. Intravenous delivery of mesenchymal stem cells in a porcine model of MI has also been reported to produce a dose-dependent beneficial effect on cardiac function. The reason for the apparent discrepancy between our study and these previous studies is unclear, but may relate to the cell type (CSCs versus bone marrow CD34^{pos} cells, cardiospheres, mesenchymal stem cells, and cardiomyocyte-like cells), animal model (rats with reperfused MI versus mice and pigs with permanent LAD ligation), and other methodological
differences. A recent study by Dawkins et al26 in a porcine model of chronic MI found that intracoronary infusion of cardiosphere-derived cells (CDCs) at escalating doses of 6.25, 12.5, and 25.0×10⁶ led to equally enhanced preservation of LV function and tissue remodeling without manifestation of a dose–efficacy relationship. That study resembles ours in the sense that in both cases, no dose dependency was found over a 4-fold range of cell numbers (0.75–3.0×10⁶ in our study and 6.25–25×10⁶ in that study).

One can only speculate as to why a 4-fold increase in cell delivery (from 0.75–3.0×10⁶) did not result in greater efficacy. We found that, regardless of the dose, the engraftment of CSCs was extremely low, implying that differentiation of transplanted cells into myocytes did not contribute importantly to the functional and structural effects of CSC therapy. We have previously found that CSCs act mainly via paracrine mechanisms,28,29 and it is unclear whether such mechanisms are dose-dependent. If the signals released by CSCs (cytokines, miRs, etc.) are amplified nonlinearly by endogenous processes (ie, if they act as triggers of endogenous repair mechanisms, such as mobilization of resident cardiac stem/progenitor cells), then it is conceivable that increased doses of CSCs may not produce a greater response because the mobilization of repair mechanisms may be unrelated to the magnitude of the stimulus.

In conclusion, this study demonstrates that intracoronary administration of syngeneic CSCs in rats with acute MI results in an improvement in LV function and structure, but the magnitude of this improvement is not dose-dependent. The threshold for efficacy is between 0.3 and 0.75×10⁶; above this threshold, the beneficial effects of CSCs on LV function and remodeling do not increase over a 4-fold range (from 0.75–3.0×10⁶). Higher doses have obvious toxic effects, as demonstrated by the increased mortality associated with the 6.0×10⁶ dose. Therefore, for practical purposes, the dose–efficacy relationship for intracoronarily administered CSCs in this model is flat. We also found that the engraftment of CSCs is negligible regardless of the dose used; even with the highest safe dose (3.0×10⁶), engraftment was insufficient to account for the benefits afforded by CSC infusion. These results have obvious implications for the design of studies in rats. If these findings can be extrapolated to humans, they would suggest that increasing the dose of CSCs above a level found to be effective and safe may not lead to increased benefit and could potentially be harmful. Given that increasing the number of cells has inherent limitations in potentiating the beneficial effects of CSC therapy, the focus of our future endeavors should be on increasing the quality and potency of the limited number of cells that can be transferred as well as defining the best individual or combinatorial cell populations to maximize the beneficial outcome of cell-based interventional strategies.

Sources of Funding

This study was funded by National Institute of Health grants P01-HL78825, 1 UM1 HL-113530, and RO1-HL74351.

Disclosures

None.


**CLINICAL PERSPECTIVE**

Although c-kit<sup>+ </sup>cardiac stem cells (CSCs) have been shown to impart salubrious effects in the setting of post-infarction heart failure, the optimal dose of these cells is unknown. To date, doses of CSCs have been chosen arbitrarily. Here we examined the effects of escalating doses of CSCs in a rat model of postinfarction cardiomyopathy. The results indicate a binary rather than a dose-related response to intracoronary administration of CSCs infused after acute myocardial infarction in this rat model. Thus, once a therapeutic effect is achieved, increasing the dose of cells does not necessarily increase the magnitude of the response, suggesting that the occurrence of beneficial remodeling may be observed only after reaching a threshold stimulus necessary to jumpstart multifaceted intrinsic myocardial reparative mechanisms. Regarding direct translational value, similar to most small animal models, this study may not be directly applicable to humans in terms of exact extrapolation of the effective cell dose used in rats. For example, the Stem Cell Infusion in Patients with Ischemic Cardiomyopathy trial suggested efficacy in humans with a dose of one million CSCs, which when measured in cells per gram of myocardium is much less than the 0.3×10<sup>6</sup> cell dose used herein, which did not show any benefit. The important concept is that once a therapeutic threshold is achieved, increasing the number of CSCs may not necessarily result in increased efficacy. Given the apparent response pattern to therapy with CSCs, this study implies that additional therapeutic benefit might be achieved by repeated lower doses of CSCs rather than a single higher individual dose.
Effects of Intracoronary Infusion of Escalating Doses of Cardiac Stem Cells in Rats With Acute Myocardial Infarction

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Circ Heart Fail. 2015;8:757-765; originally published online May 20, 2015; doi: 10.1161/CIRCHEARTFAILURE.115.002210

Circulation: Heart Failure is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/8/4/757

Data Supplement (unedited) at:
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**Supplemental Figure 1**

Myocardial collagen content assessed from LV sections stained with picrosirious red. Quantitation was performed by expressing collagen content as percent of total area in the scar (risk) and noninfarcted (remote) region. Collagen content in the risk region was reduced by CSC administration at a dose of 0.75 M or higher ($P<0.05$ vs. vehicle for all groups), but not at the dose of 0.3 M; Note that the reduction did not exhibit a dose-dependent pattern. None of the CSC doses had any effect on collagen in the remote region. Data are means ± SEM.
Supplemental Figure 2

Analysis of capillary density by isolectin staining. (A) Representative confocal microscopic images from a vehicle, 0.75 M, 1.5 M, and 3.0 M CSC-treated heart (the images were taken from the border zone) showing FITC-conjugated isolectin B4-stained vessels. (B) Quantitative analysis of isolectin positive cells in the risk and remote (noninfarcted) region. Isolectin-stained capillaries averaged approximately 800/mm² both in the risk and remote regions of the vehicle-treated hearts. Three escalating doses of CSCs (0.75, 1.5, and 3.0 M) dose-dependently increased capillary density in both the risk and remote regions; compared with vehicle-treated hearts, the density was significantly higher in the 1.5 M and 3.0 M groups in the risk region, and in the 3.0 M group in the remote region. Data are means ± SEM.