Repeated Administrations of Cardiac Progenitor Cells Are Markedly More Effective Than a Single Administration: A New Paradigm in Cell Therapy

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Abstract

Rationale—The effects of c-kit^{POS} cardiac progenitor cells (CPCs) (and adult cell therapy in general) on left ventricular (LV) function have been regarded as modest or inconsistent.

Objective—To determine whether three CPC infusions have greater efficacy than one infusion.

Methods and Results—Rats with a 30-day-old myocardial infarction received one or three CPC infusions into the LV cavity, 35 days apart. Compared with vehicle-treated rats, the single-dose group exhibited improved LV function after the 1st infusion (consisting of CPCs) but not after the 2nd and 3rd (vehicle). In contrast, in the multiple-dose group regional and global LV function improved by a similar degree after each CPC infusion, resulting in greater cumulative effects. For example, the total increase in LV ejection fraction was approximately triple in the multiple-dose group vs. the single-dose group (*P*<0.01). The multiple-dose group also exhibited more viable tissue and less scar, less collagen in the risk and noninfarcted regions, and greater myocyte density in the risk region.

Conclusions—This is the first demonstration that repeated CPC administrations are markedly more effective than a single administration. The concept that the full effects of CPCs require repeated doses has significant implications for both preclinical and clinical studies; it suggests that the benefits of cell therapy may be underestimated or even overlooked if they are measured after a single dose, and that repeated administrations are necessary to properly evaluate the effectiveness of a cell product. In addition, we describe a new method that enables studies of repeated cell administrations in rodents.

Keywords

Cell therapy; cardiac progenitor cells; myocardial infarction; ischemia reperfusion injury; left ventricular function; left ventricular remodeling

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DISCLOSURES
None.

INTRODUCTION

Although administration of c-kit[POS] cardiac progenitor cells (CPCs) has produced encouraging results in preclinical animal models of ischemic cardiomyopathy and in a phase I clinical trial,[1–11] the effects of these cells have been regarded as modest or disappointing, and their therapeutic utility (and that of adult cell therapy in general) questioned.[12–16] A major factor limiting the benefits of CPC therapy is the poor engraftment of transplanted cells. We have demonstrated that, following intracoronary or intramyocardial injection of syngeneic CPCs in mice, rats, and pigs, the number of cells remaining in the heart declines precipitously to very low values[4–7, 17–20]; for example, <8% of the CPCs present immediately after transplantation remain in the mouse heart one week later, and after 35 days this number is <3%.[17, 18] Rapid disappearance of transplanted cells has also been observed with most other cells types,[1, 2, 21–31] indicating that poor engraftment is a universal problem in cell therapy. Thus, strategies that increase the presence of CPCs in the heart may greatly enhance the therapeutic benefits of these cells and, conceivably, other cell types as well.

One approach that may overcome, in part, the problem of poor engraftment and augment the myocardial content of CPCs (thereby augmenting therapeutic efficacy) is to perform multiple CPC injections. Even if after subsequent injections the cells disappear at the same rate at which they disappear after the first, each injection should result in a transient spike in the number of CPCs in the tissue, such that the average density of these cells over time should be higher. Surprisingly, no previous study has been performed to evaluate the effects of repeated CPC administrations. More generally, in the field of cell therapy at large, the overwhelming majority of the studies performed heretofore have evaluated efficacy on the basis of a single treatment; very few studies[32–36] have used repeated doses. The problem with using a single dose is that, since the myocardial content of progenitor cells declines rapidly after transplantation, irrespective of which cell type is used[1, 2, 4–7, 17–31], injecting a cell product only once may not be an adequate test of the efficacy of that product; for the therapeutic effects to become apparent, repeated doses may be necessary to replace the cells that die after transplantation. Just as most pharmacologic agents are ineffective when given once but can be highly effective when given repeatedly, so a cell product that appears to be ineffective or modestly effective when given as a single treatment may, in fact, turn out to be quite efficacious if given repeatedly. If this concept is correct, the current paradigm of cell therapy would change dramatically.

The goal of the present investigation was to test this hypothesis. Specifically, we sought to determine whether three administrations of CPCs, performed 35 days apart, result in greater improvement in left ventricular (LV) structure and function compared with a single CPC administration. Because ischemic heart failure appears to be the clinical setting in which cell therapy offers the greatest promise,[1, 2] we used a rat model of ischemic cardiomyopathy caused by an old myocardial infarction (MI), in which CPC administration has previously been found to be beneficial.[6] Our finding that three doses of CPCs are markedly more effective than a single dose has potentially far-reaching implications for cell therapy.

METHODS

A detailed Methods section is included in the online supplement.

Isolation, culture, and transfection of c-kit[POS] CPCs

CPCs were isolated from the entire heart (atria and ventricles) of adult male Fischer 344 rats as described[4] and phenotyped by FACS analysis of fixed cells. The cells were injected into rat hearts at passages 6 to 8. The rationale for using male donor CPCs was that the fate of transplanted CPCs can be tracked by detecting Y-chromosome^{pos} cells, although previous studies have suggested that bone marrow-derived mesenchymal stromal cells differ significantly between male and female sources, with female cells usually performing more robustly.[37]

Surgical procedures, treatment protocol, and preliminary studies

The rat model of old MI has been described previously.[6] Adult female Fischer 344 rats (age, 4 months; weight 175 ± 2 g) underwent a 2-h occlusion of the left anterior descending coronary artery followed by reperfusion. Thirty days after surgery, the animals were randomly allocated to three treatment groups: vehicle (control), single dose, or multiple doses (Fig. 1). Randomization was performed using the MS Excel random group generator.

All rats received an echo-guided intraventricular injection, which was performed using the Vevo 2100 Imaging System (VisualSonics) equipped with a 20-MHz transducer, a Vevo Image Station with Injection Mount and micro-manipulation controls, and a Chemyx NanoJet Stereotaxic syringe pump (Chemyx Inc. TX). Before the procedure, rats were anesthetized with 3% isoflurane. The anterior chest was shaved and the animals were placed on the imaging table in the right lateral decubitus position with the left lateral side facing the injection mount. Light anesthesia was maintained with 1% isoflurane. With the imaging transducer aligned perpendicularly to the injection mount, the left ventricle was initially imaged in the parasternal plane and a good 2D long-axis view was procured by adjusting the angle and position of the imaging table. The transducer was then turned 90 degrees clockwise and the left ventricle was scanned in the 2D short-axis and color Doppler views from apex to base to determine the optimal site for needle insertion, a site that did not include the infarct scar or a coronary artery. Under the guidance of a real-time B-mode view, a 30 G injection needle (1" length) was then carefully inserted from the left lateral side using the micro-manipulation controls and advanced into the center of the LV cavity.

Rats received an infusion of CPCs (12×10^6 cells in 5 ml of DPBS (Dulbecco's phosphate-buffered saline, Gibco) or vehicle (5 ml of DPBS) into the LV cavity with the injection pump at a rate of 1.25 ml/min for 4 min. The single-dose and multiple-dose groups received CPCs whereas the vehicle group received DPBS. Thirty-five days later, this procedure was repeated; rats received a second infusion of CPCs (multiple-dose group) or an infusion of DPBS (single-dose and vehicle groups). Thirty-five days later, rats received a third infusion of CPCs (multiple-dose group) or another infusion of DPBS (single-dose and vehicle groups). mCherry-labeled CPCs were used for the 1st treatment, non-labeled CPCs for the 2nd treatment, and GFP-labeled CPCs for the 3rd treatment. To monitor formation of new

cells, in all groups 5-bromo-2'-deoxyuridine (BrdU, Sigma) was given for 35 days after the 1st treatment and 5-iodo-2'-deoxyuridine (IdU, Sigma) for 35 days after the 3rd treatment (both were given in the drinking water at a final concentration of 0.1%). At 35 days after the 3rd treatment, rats were subjected to hemodynamic studies and euthanized for histologic studies or PCR analysis (Fig. 1).

Before initiating the protocol, pilot studies were conducted to identify a dose of CPCs that would results in LV myocardial retention similar to that observed after intracoronary infusion of 1 million CPCs, the dose used in our previous studies in rats.[3, 4, 6] Rats underwent a 90-min coronary occlusion and reperfusion. Four hours after reperfusion, they received 1.0×10^6 CPCs (in 1 ml of DPBS) via intracoronary infusion as in our previous studies[3–6], 3.0, 9.0, or 12.0×10^6 CPCs (in 5 ml of DPBS over 4 min) using echo-guided injection into the LV cavity, or 3.0×10^6 CPCs i.v. Rats were euthanized 24 h after CPC delivery and the heart was harvested to measure the number of CPCs in the tissue by real-time PCR.

Echocardiographic studies

All echocardiographic analyses were performed by investigators who were blinded to treatment allocation. Serial echocardiograms were obtained at five time-points: baseline (three days before coronary artery occlusion), 30 days after MI (before the 1st treatment), 35 days after the 1st treatment (before the 2nd treatment), 35 days after the 2nd treatment (before the 3rd treatment), and 35 days after the 3rd treatment (before euthanasia)(Fig. 1). The echocardiographic studies were performed as described[3–6] using a Vevo 2100 Imaging System equipped with a 20-MHz transducer.

Hemodynamic studies

All hemodynamic analyses were performed by investigators who were blinded to treatment allocation. The hemodynamic studies were conducted 35 days after the 3rd treatment, just before euthanasia (Fig. 1). The protocol has been described.[4–6]

Histologic studies

The protocol for histologic analyses has been described.[4–7, 38]

Immunohistochemistry was performed in formalin-fixed, paraffin-embedded, 4-μm-thick heart sections. To assess the fate of the transplanted male CPCs, single-labeled Y chromosome was detected by fluorescence *in situ* hybridization (FISH) according to the modified manufacturer's protocol (ID Labs, London, ON).[4, 39]

Measurement of transplanted cells by real-time PCR

To determine the absolute number of transplanted CPCs, a quantitative real-time PCR-based method was used as previously described[17, 18] in a subset of hearts from the single-dose group (n=8) and the multiple-dose group (n=11), which were frozen just after excision.

Statistical analysis

All data are expressed as means ± SEM. Echocardiographic data were analyzed with two-way repeated-measures ANOVA followed by Student's t-tests with Bonferroni correction for intra- and inter-group comparisons, as appropriate. All parametric data including morphometric, histologic, immunohistochemical, and hemodynamic data were analyzed by one-way ANOVA followed by Student's t-tests with Bonferroni correction for inter-group comparisons.[40–42] Mortality was analyzed by the chi-square test. All analyses were conducted with SigmaStat 3.5. P values <0.05 were considered significant.

RESULTS

A total of 104 rats were included in this study: 19 for the preliminary dosing studies and 85 for the final protocol.

Characterization of CPCs

Supplementary Figures IIA and IIB are representative confocal images of mCherry- and GFP-labeled c-kit[POS] CPCs. Similar to our previous studies[4, 5], FACS analysis showed that 82.9 ± 2.2% (n=7) of the cells were c-kit positive at the passage when they were injected (passages 6–8); in addition, 80.8 ± 5.4% (n=5) were mCherry positive and 97.6 ± 0.4% (n=2) GFP positive (Supplementary Figs. IIC and D). CPCs were negative for CD45 (0.5 ± 0.1%, n=5) and CD31 (0.4 ± 0.0%, n=5), and expressed mesenchymal markers (CD 90, 19.7 ± 9.8%, n=5; CD105, 68.1 ± 6.5%, n=5; CD73, 92.2 ± 4.6%, n=5; CD29, 99.5 ± 0.1%, n=5) (Supplementary Fig. IID). The mean population doubling time was 28.9 ± 2.8 h (n=5).

Preliminary studies

We have previously found that 1×10^6 CPCs, delivered intracoronarily, produce an improvement in LV function in this rat model.[6] Preliminary studies were conducted in 19 rats to select a dose of CPCs that would result in a comparable degree of myocardial retention when delivered into the LV cavity. As shown in Table 1, intraventricular infusion of 3×10^6, 9×10^6, and 12×10^6 CPCs resulted, 24 h later, in a dose-dependent retention of cells. Since the number of CPCs retained 24 h after the 12×10^6 dose (112,983±56,300 cells/heart, n=6) was similar to that retained 24 h after intracoronary infusion of 1×10^6 CPCs (118,924±24,458, n=3), and since, as mentioned above, the latter treatment is effective in enhancing LV function in this rat model[6], we selected 12×10^6 CPCs as the dose to be used in the present study. The equivalency of the intraventricular CPC dose used herein and the intracoronary CPC dose used previously is further supported by the fact that the benefits of 12×10^6 CPCs in the single-dose group (*vide infra*) were comparable to those observed previously in this same model after intracoronary infusion of 1×10^6 CPCs.[6] In contrast to the intraventricular infusion, no CPCs could be found in the heart after intravenous infusion of 3×10^6 CPCs (Table 1).

Exclusions and gross measurements

As shown in Supplementary Table I, of the 85 rats that were subjected to coronary artery occlusion/reperfusion, 13 died within seven days after MI. At 30 days after MI, the remaining 72 rats were assigned to one of the three treatment groups (vehicle, single dose, or

multiple doses); nine of these animals died after CPC infusion because of intrathoracic bleeding from the LV injection site. Thus, a total 63 rats completed the protocol; of these, two were excluded because of prespecified criteria (one because LV EF decreased <15 units after MI and before treatment, as shown by echocardiography [Supplementary Fig. IA] and one because of small scar size [<10% of risk region], as shown by trichrome stain [Supplementary Fig. IB]). Therefore, a total of 61 rats (16 in the vehicle group, 20 in the single-dose group, and 25 in the multiple-dose group) were included in the final analysis. Of these, 50 rats (16 in the vehicle, 16 in the single-dose, and 18 in the multiple-dose group) underwent echocardiographic and hemodynamic studies, 42 (16, 12, and 14, respectively) were used for pathologic and immunohistochemical analyses, and 8 (4 in the single-dose and 4 in the multiple-dose group) were used for qPCR analysis of CPC retention. An additional 11 rats (4 in the single-dose group and 7 in the multiple-dose group) were used for qPCR analysis without echocardiographic or hemodynamic analyses; two of these rats (in the multiple-dose group) were excluded because the CPC counts were >2 SD above the average, likely as a result of accidental injection of cells into the LV wall. Therefore, data from 17 rats (8 in the single-dose and 9 in the multiple-dose group) were used for the qPCR analysis of CPC retention (Supplementary Table I).

There were no significant differences in body weight among the three groups throughout the experimental protocol (Supplementary Table II). Similarly, there were no significant differences among the groups with respect to LV weight or LV volume measured at postmortem examination (Supplementary Table II).

Echocardiographic measurements

Echocardiographic measurements are summarized in Supplementary Table III; representative echocardiographic recordings are illustrated in Supplementary Fig. III. Before the 1st treatment (30 days after MI), LV end-diastolic volume (LVEDV) and end-systolic volume (LVESV) were markedly increased from baseline (Fig. 2A), whereas diastolic thickness and systolic thickening fraction (ThF) in the infarcted wall (Fig. 3A) and LV EF (Figs. 4A and B) were markedly decreased. There were no significant differences among the vehicle, single-dose, and multiple-dose groups in any of these variables, indicating that before the 1st treatment, the severity of post-MI LV remodeling and dysfunction was comparable in all groups.

After the 1st treatment, however, the three groups exhibited a different course. As expected, the vehicle-treated (control) group continued to show progressive deterioration of both regional and global LV function (Figs. 2–4; Supplementary Fig. III). Thus, in the 105-day interval between the 1st treatment and euthanasia, LVESV increased progressively compared with pretreatment values (at 30 days after MI) (Fig. 2B), whereas diastolic thickness of the infarcted wall (IWTd) (Fig. 3B), systolic ThF in the infarcted wall (Fig. 3B), and LV EF (Fig. 4D) all decreased progressively.

The single-dose group exhibited a significant improvement in indices of regional and global LV function after the 1st infusion (which consisted of CPCs); however, no further significant improvement was observed after the 2nd and 3rd infusions (which consisted of vehicle) (Figs. 2–4). For example, compared with the vehicle group, administration of CPCs in the single-

dose group resulted, 35 days later, in a decrease in LVESV (Figs. 2A and B) and in the percentage of the LV circumference that was akinetic (akinetic length) (Figs. 3C and D) and in an increase in the diastolic thickness in the infarcted wall (Figs. 3A and B), in the ThF in the infarcted LV wall (Figs. 3A and B), as well as in LV EF (Fig. 4); after the 2nd and 3rd infusions (vehicle), none of these variables changed significantly (Figs. 2–4).

In contrast, the multiple-dose group exhibited a progressive improvement in regional and global LV function after each CPC infusion (Figs. 2–4). Each additional treatment produced additional benefit, and the magnitude of the improvement observed after the 2nd and 3rd infusions was roughly comparable to that observed after the 1st infusion (Figs. 2–4). Thus, compared with pretreatment values (at 30 days after MI), the diastolic thickness of the infarcted wall increased by 0.10 ± 0.02 mm, 0.20 ± 0.02 mm, and 0.32 ± 0.03 mm after the 1st, 2nd, and 3rd infusion of CPCs, respectively ($P < 0.01$ for 2nd vs. 1st and 3rd vs. 2nd) (Fig. 3B); systolic ThF in the infarcted wall increased by $8.5 \pm 2.4\%$, $16.5 \pm 2.8\%$, and $22.3 \pm 3.9\%$ ($P < 0.05$ for 2nd vs. 1st and 3rd vs. 2nd)(Fig. 3B); LVESV decreased by 10.2 ± 4.0 μL, 18.2 ± 3.5 μL, and 24.2 ± 5.5 μL ($P < 0.05$ for 2nd vs. 1st) (Fig. 2B); LV EF increased by $3.4 \pm 0.5\%$, $7.4 \pm 0.9\%$, and $13.0 \pm 0.8\%$ ($P < 0.01$ for 2nd vs. 1st and 3rd vs. 2nd) (Fig. 4D); and the percentage of the LV circumference that was akinetic (akinetic length) decreased by $5.6 \pm 2.6\%$, $10.5 \pm 2.4\%$, and $13.6 \pm 2.0\%$ (Fig. 3D).

As a result of this stepwise improvement, the cumulative beneficial effects of cell therapy after three CPC infusions were much greater than the effects seen after one CPC infusion. For example, the total (cumulative) increase in LV EF between pretreatment and end of the study in the multiple-dose group ($13.0 \pm 0.8\%$) was approximately triple that in the single-dose group ($4.7 \pm 0.8\%$) ($P < 0.01$) (Fig. 4D). The total (cumulative) increase in infarcted wall ThF was ~2.5 greater in the former (22%) than in the latter (9%) ($P < 0.05$; Fig. 3B). Similarly, the total (cumulative) decrease in akinetic length was ~2.5 times greater in the multiple-dose than in the single-dose group (-13.6% vs. -5.6%, $P < 0.05$) (Fig. 3D). The greater therapeutic efficacy of multiple CPC infusions is further demonstrated by the fact that, at the end of the protocol, there were statistically significant differences in infarcted wall thickness, infarcted wall ThF, LV EF, LVESV, and akinetic length not only between the multiple-dose group and the vehicle group, but also between the multiple-dose group and the single-dose group ($P < 0.01$ for all comparisons) (Figs. 2–4). An additional difference is that in the multiple-dose group, the systolic ThF in the posterior (noninfarcted) LV wall was significantly greater than in the vehicle group after the 2nd and 3rd treatment ($P < 0.01$); in contrast, in the single-dose group systolic ThF in the posterior wall was significantly ($P < 0.01$) greater than in the vehicle group only after the 3rd treatment (Fig. 3A).

For LV EF, repeated-measures two-way ANOVA demonstrated a significant interaction between time and group (F=35.57, $P < 0.001$), indicating that the relationship between LV function and time depended on treatment group. Similar conclusions were achieved for LVESV, LV stroke volume, diastolic thickness of the infarcted wall, systolic ThF in the infarcted wall, and akinetic length (Figs. 2 and 3). Trend analysis indicated that i) in the vehicle group, LV EF declined significantly over time ($P < 0.001$); ii) in the single-dose group, a single linear trend could not capture the EF profile over time because EF increased after the 1st treatment ($P < 0.001$) but plateaued after the 2nd and 3rd treatment; and iii) in the

multiple-dose group, LV EF increased significantly over time (*P*<0.001) and the LV EF profile could be captured by a linear trend. Similar conclusions were achieved when trend analysis was used for the other variables illustrated in Figs. 2 and 3.

In summary, the patterns observed in the three treatment groups were quite different: indices of regional and global LV function improved after each treatment (by a similar amount) in the multiple-dose group, improved only after the 1st treatment in the single-dose group, and either worsened or were unchanged after each treatment in the vehicle group.

Echocardiography did not demonstrate any significant effect of CPCs on LV dilatation. As shown in Fig. 2A, LVEDV remained relatively stable in vehicle-treated rats during the three treatments and was not affected by either single or multiple administrations of CPCs (Fig. 2A). The observation that CPCs did not reverse LV dilatation is consistent with our previous studies in this rat model of old MI.[6]

Hemodynamic measurements

The hemodynamic studies, which were performed just before euthanasia, also demonstrated superior LV function in the multiple-dose group compared with the single-dose group (Figs. 5A and B; Supplementary Table IV). Both of these groups exhibited a significant improvement in most hemodynamic parameters compared with the vehicle group; however, the efficacy of multiple treatments was superior to that of a single treatment, as demonstrated by the fact that LV EF (a load-dependent index of LV systolic function) and preload recruitable stroke work and end-systolic elastance (two load-independent indices) were significantly greater in the multiple-dose vs. the single-dose group, whereas ESV was significantly smaller (Fig. 5B). Thus, two independent methods of functional assessment (echocardiography and hemodynamic studies with a conductance catheter) consistently demonstrated that multiple administrations of CPCs produced a greater improvement in regional and global LV function compared with a single administration.

Morphometric and histological analysis

As illustrated in Fig. 6A, the vehicle group exhibited LV dilatation and an extremely thin infarcted wall with a confluent scar. In the single-dose and multiple-dose groups, the infarcted wall was thicker and the amount of viable tissue within the risk region was greater compared with the vehicle group (Fig. 6A).

In each heart, a detailed quantitative analysis was performed on two sections (one from each of two mid-ventricular slices); the results are summarized in Fig. 6B and Supplementary Table V. Although the size of the risk region was similar among the three groups, both the single-dose and the multiple-dose groups exhibited a greater amount of viable myocardium within the risk region (60.6 ± 2.2% and 67.7 ± 2.9% of the risk region, respectively) than the vehicle group (51.4 ± 2.3%, *P*<0.05 vs. the single-dose group and *P*<0.01 vs. the multiple-dose group). Unlike the single-dose group, however, the multiple-dose group also exhibited a significant increase in the thickness of the infarcted wall and in the percentage of LV weight that was accounted for by viable tissue (*P*<0.05 and <0.01, respectively, vs. the vehicle group) (Fig. 6B). There was a trend for the percentage of scarred tissue to be smaller and the infarcted wall thickness and percentage of viable myocardium to be greater in the

multiple-dose compared with the single-dose group, but the differences were not statistically significant (Fig. 6B). Taken together, the morphometric data confirm the echocardiographic finding of greater infarcted wall thickness in the multiple-dose than in the single-dose group (Fig. 3A) and reveal a difference between single and multiple treatments with respect to the amount of viable myocardium: both treatments increased viable tissue and decreased scar within the risk region, but when these variables were expressed as percentages of the entire left ventricle, only multiple treatments were associated with an increase in the total amount of viable tissue and a decrease in the total scar burden.

Myocardial fibrosis plays a key role in the pathology of LV remodeling after MI.[43] In the noninfarcted region, collagen content was significantly less in the multiple-dose compared with the single-dose group (P<0.05) (Figs. 6C and D). This reduced collagen deposition in the myocardium may have contributed, at least in part, to the functional benefits of CPC therapy.

Myocyte cross-sectional area and myocyte and vessel density

Myocyte cross-sectional area and myocyte density were assessed by staining myocytes with an anti-alpha-sarcomeric actin (α-SA) antibody and myocyte membranes with FITC-conjugated WGA to facilitate counting individual myocytes (Fig. 7 and Supplementary Fig. IV). In the noninfarcted region, there were no significant differences among groups (Figs. 8B and C). However, in the risk region myocyte cross-sectional area was significantly smaller and myocyte density was significantly greater in the two treated groups compared with the vehicle group (Figs. 7B and C); in the multiple-dose group, these changes were more pronounced, such that both myocyte cross-sectional area and density were significantly different from the single-dose group (P<0.05) (Figs. 7B and C). Consistent with these data, analysis of the distribution of cardiomyocyte cross-sectional area demonstrated no difference in the noninfarcted region but a shift to the left in the risk region in the single-dose group and, even more so, in the multiple-dose group (Supplementary Fig. IV). Although other explanations are possible, increased myocyte density is consistent with increased formation of new myocytes.

Vascular density was assessed by staining tissue sections with FITC-conjugated Isolectin B4 (Supplementary Fig. V). In all groups, vascular density was significantly less in the risk region than in the noninfarcted region, but no differences were noted among the three groups.

Survival of transplanted CPCs

Survival of transplanted CPCs was assessed by two independent methods. The CPCs administered during the 1st infusion were labeled with mCherry and those infused during the 3rd infusion with GFP (Fig. 1). As shown in Table 2, at the end of the study (35 days after the 3rd CPC infusion and 105 days after the 1st infusion), very few GFP[POS] cells and even fewer mCherry[POS] cells were found in the left ventricle using immunohistochemistry (n=14). In addition, the number of CPCs remaining in the heart at the end of the protocol was measured using quantitative real-time PCR in two subsets of rats (Supplementary Table VI). Transplanted CPCs were detected only in five of the eight rats analyzed in the single-

dose group and nine of the eleven rats analyzed in the multiple-dose group. In the hearts with detectable CPCs, the number of these cells was extremely low: 3,330±1,451 in the entire heart in the single-dose group (n=5) and 5,078±1,409 in the multiple-dose group (n=7) (*P*=NS). Assuming that no cells were present in hearts with no detectable CPCs, the total content of CPCs averaged 2,081 ± 1,060 cells in the single-dose group (n=8) and 3,950±1,310 in the multiple-dose group (n=9) (*P*=NS). Thus, the number of transplanted cells that were engrafted in the heart was very low; it tended to increase after three CPC infusions vs. a single infusion, but the difference was not statistically significant, possibly because of the large variability.

Analysis of cell proliferation

In previous studies, we have observed a pronounced and prolonged proliferative response to a single CPC administration.[4, 6] To determine the effect of multiple CPC doses on cell proliferation, rats were given BrdU for 35 days after the 1st treatment (days 1–35 after start of therapy) and IdU for 35 days after the 3rd treatment (days 71–105 after start of therapy) (Fig. 1). In all three groups, the number of BrdU^{POS} and IdU^{POS} cells was significantly greater in the risk region than in the noninfarcted region (Figs. 8C–F), indicating greater cell proliferation and/or greater infiltration of proliferating cells in the former - a finding consistent with our previous observations.[4, 6]

In the noninfarcted region, there were no significant differences among the three treatment groups with respect to either BrdU^{POS} or IdU^{POS} cells (Fig. 8C–F). In contrast, significant differences were noted in the risk region. In vehicle-treated hearts, 13.8 ± 1.0% of nuclei in the risk region were BrdU^{POS} (Fig. 8C) and 22.2 ± 1.9% IdU^{POS} (Fig. 8D) at the end of the study, indicating that a substantial number of new cells were formed between days 1–105 (BrdU^{POS} cells) and between days 71–105 after the beginning of treatment (IdU^{POS} cells). These values are consistent with our previous results.[4, 6]

A single dose of CPCs did not result in a significant increase in BrdU^{POS} cells (Fig. 8C); this, however, does not rule out a proliferative response to CPCs because BrdU was given only for the first 35 days after CPC infusion and cells may have proliferated early after CPC infusion and then died by the time rats were euthanized 105 days later. A single dose of CPCs did produce a significant increase in the number of IdU^{POS} cells (Fig. 8D), indicating that at 71–105 days after the administration of CPCs, there was increased cell proliferation. The ability of a single CPC infusion to promote cell proliferation several months later is consistent with our previous findings in this rat model.[4, 6] The number of BrdU^{POS}/IdU^{POS} (double positive) cells was not significantly increased 105 days after a single CPC administration (Fig. 8E), but again, an early proliferative response may have been missed if significant numbers of BrdU^{POS} cells died before euthanasia. This possibility is supported by the fact that, in the vehicle-treated group, the number of IdU^{POS} cells (22.2 ± 1.9 % in risk region and 14.6 ± 0.8 % in noninfarcted region) was greater than the number of BrdU^{POS} cells (13.8 ± 1.0 % in risk region and 10.8 ± 1.0 % in noninfarcted region) (Figs. 8C and D).

In rats that received multiple doses of CPCs, the number of BrdU^{POS} cells was markedly increased vs. both the single-dose group and the vehicle group (Fig. 8C), suggesting that the

2nd and 3rd infusions of CPCs induced a robust proliferative response (on days 36–105 after the beginning of CPC treatment). The number of IdU^{POS} cells was significantly increased vs. the vehicle group but not vs. the single-dose group (Fig. 8D), suggesting that while three CPC doses produced a robust proliferation on days 71–105 after the beginning of treatment, this proliferation was not greater than that induced by a single treatment given earlier. Unlike the single-dose group, the multiple-dose group exhibited a significant increase in the number of BrdU^{POS}/IdU^{POS} (double positive) cells (Fig. 8E), which constituted ~1/2 of all IdU^{POS} cells (Fig. 8D), suggesting that with repeated CPC infusions, ~1/2 of the new cells formed on days 71–105 after the beginning of treatment were derived from cells formed on days 1–70. When all newly-formed (BrdU^{POS} or IdU^{POS}) cells were combined, their number was significantly increased after both a single and multiple CPC infusions (Fig. 8F); the increase tended to be greater after multiple CPC infusions, but the difference vs. the single-dose group was not significant.

Taken together, these data indicate that a single CPC infusion promoted a sustained proliferative response in the risk region (or a greater infiltration of inflammatory cells in the risk region) that was still evident 2–3 months (71–105 days) later, and that multiple CPC infusions tended to further augment this response.

Analysis of new myocyte formation

Formation of new mature myocytes was assessed by measuring the number of BrdU^{POS} or IdU^{POS} cells that expressed cTnI and exhibited a morphology typical of adult myocytes (Fig. 8). (Thus, the small cells that express alpha-sarcomeric actin but lack the morphology of mature cardiomyocytes [cells that we observed after CPC transplantation in our previous studies[4, 6]] were not included in this analysis.)

In Fig. 8, the number of new mature myocytes (BrdU^{POS}/cTnI^{POS} or IdU^{POS}/cTnI^{POS} cells) is expressed both as a percent of total nuclei (Figs. 8G–J) and as a percent of total myocytes (Figs. 8K–N). In all groups, these cells tended to be more abundant in the noninfarcted than in the risk region but, in absolute terms, they were rare. For example, in the vehicle-treated group, the number of BrdU^{POS} or IdU^{POS} mature myocytes was <0.5% of the nuclei and <0.5% of myocytes both in the risk region and in the noninfarcted region (Fig. 8G–N). The density of these cells was not significantly increased by a single CPC infusion (Fig. 8G–N). Three CPC infusions were associated with a significant increase in BrdU^{POS} and IdU^{POS} mature myocytes in the risk region (P<0.05 vs. vehicle-treated rats) (Figs. 8G–N), but even in this group, the percentage of these cells was very low (<0.5% of nuclei and <2% of myocytes in the risk region and <1 % of nuclei and <1% of myocytes in the noninfarcted region).

Taken together, these data indicate that the number of newly-formed mature myocytes was very small in the absence of CPC infusion and did not change after a single infusion; although this number increased significantly after three CPC infusions, it was still extremely low in absolute terms, and thus not sufficient to account for the improvement in function.

Analysis of Y-chromosome[POS] cells

To assess the fate of transplanted male CPCs, the number of Y-chromosome^{pos} cells was measured by FISH. The advantage of using the Y-chromosome to track transplanted cells in long-term studies is that, unlike EGFP or beta-galactosidase, its presence is not affected by changes in gene expression.[44–46] Y-chromosome^{pos} cells were detected both in the single-dose and in the multiple-dose groups, but they were significantly more abundant in the multiple-dose group, both in the risk and in the noninfarcted region (Fig. 9C). The vast majority (>90%) of Y-chromosome^{pos} cells, however, did not show a myocyte morphology: in the risk region, 90.7 ± 2.0 and $93.5 \pm 1.4\%$ of Y-chromosome^{pos} cells were nonmyocytes in the single- and multiple-dose groups, respectively; in the noninfarcted region, $71.4 \pm 7.2\%$ and $78.4 \pm 6.0\%$ were nonmyocytes. Consequently, the number of Y-chromosome[pos] myocytes was minuscule both in the single-dose and in the multiple-dose groups: $0.25 \pm 0.05\%$ and $0.37 \pm 0.07\%$ of total nuclei, respectively, in the risk region, and $0.25 \pm 0.11\%$ and $0.54 \pm 0.17\%$, respectively, in the noninfarcted region (Fig. 9D). Similar values were obtained when Y-chromosome[pos] myocytes were expressed as a percentage of total myocytes: they accounted for <1% of myocytes in the risk region and <0.5% in the noninfarcted region (Fig. 9E).

Taken together, these data indicate that, regardless of the number of CPC infusions, very few transplanted cells (or their progeny) engraft in the heart and, of these, only a very small fraction differentiate into mature cardiomyocytes. Multiple CPC infusions produce an increase in CPC engraftment that is statistically significant but, in absolute terms, is quite small.

Analysis of BrdU and IdU positivity revealed that only a fraction of the Y-chromosome[pos] myocytes had incorporated BrdU or IdU (Figs. 9F–K). For example, in the noninfarcted region of the multiple-dose group, Y-chromosome[pos] myocytes accounted for $0.54 \pm 0.17\%$ of all nuclei (Fig. 9D), whereas Y-chromosome[pos]/BrdU[POS] myocytes accounted for $0.04 \pm 0.04\%$ of all nuclei and Y-chromosome[pos]/IdU[POS] myocytes for $0.09 \pm 0.06\%$ of all nuclei (Fig. 9H). This finding suggests that not all CPCs underwent cell cycling before differentiating into mature myocytes.

DISCUSSION

The safety and efficacy of repeated CPC administrations are unknown because all previous studies have delivered these cells only once. The salient findings of the present investigation can be summarized as follows: i) in a rat model of chronic ischemic cardiomyopathy, three CPC infusions performed 35 days apart produced a markedly greater improvement in both regional and global parameters of LV function compared with a single infusion; the superiority of multiple treatments was consistently observed with two independent techniques (echocardiography and hemodynamic studies); ii) both echocardiography and morphometry showed that the infarcted LV wall was significantly thicker in the multiple-dose group than in the vehicle group; iii) unlike one CPC infusion, three CPC infusions resulted in a significant decrease in the total LV scar burden, a significant increase in total LV viable tissue, and a significant decrease in collagen content in the noninfarcted region; iv) both a single and multiple CPC treatments promoted sustained cellular proliferation (or

infiltration of inflammatory cells) in the risk region, but this effect tended to be more pronounced in the latter group; v) despite the remarkable functional benefits conferred by CPC infusion, the number of transplanted cells remaining in the heart at the end of the study was minuscule ($\approx$ 2,000 cells/heart in the single-dose group and $\approx$ 4,000 cells/heart in the multiple-dose group); vi) regardless of the number of CPC infusions, very few (<10%) of the transplanted cells that remained in the heart at the end of the study differentiated into mature cardiomyocytes, and <1% of all myocytes were derived from transplanted cells ; vii) both a single and multiple CPC infusions resulted in a decrease in myocyte cross-sectional area and an increase in myocyte density in the risk region, but these changes were significantly greater in the multiple-dose group; viii) unlike a single CPC infusion, multiple CPC infusions were associated with a significant increase in new ($BrdU^{POS}$ or IdU^{POS}) mature myocytes in the risk region vs. vehicle-treated rats; however, even after multiple CPC infusions, the absolute number of newly-formed ($BrdU^{POS}$ or IdU_{POS}) myocytes was quite small (<1% of total nuclei and <2% of total myocytes).

Taken together, these results demonstrate that three repeated doses of CPCs are safe and considerably more effective in improving LV performance and structure than a single CPC dose, and that these effects cannot be ascribed to differentiation of transplanted cells into myocytes. Many previous investigations have examined the effects of various cells types in models of ischemic cardiomyopathy.[2] However, to our knowledge, this is the first study to examine the effects of repeated administrations of CPCs or any cardiac-derived cell type.

One of the most important findings of this study is that each CPC infusion was associated with a similar degree of improvement in regional and global LV function. For example, systolic ThF in the infarcted region (an index of regional function in this region) increased by 9 ThF units after the 1st CPC infusion, 8 units after the 2nd, and 5 units after the 3rd (Fig. 3B). LVEF increased by 3.4 units after the 1st CPC infusion, 4.0 units after the 2nd, and 5.5 units after the 3rd (Fig. 4C). These results suggest that each dose of CPCs produces a comparable improvement in LV function, at least within the first three treatments. Whether additional (>3) doses also produce similar effects remains to be determined. Nevertheless, the fact that the magnitude of benefit is relatively constant after each of the first three treatments implies that the potential benefits of cell therapy may be underestimated or even overlooked if they are measured after a single administration of cells, and that repeated administrations are necessary to properly evaluate the effectiveness of a cell product. For example, in this study the first CPC administration produced a modest (3.4%) increase in LVEF (Fig. 4C). Depending on the experimental model and the power of the study, such a small increase may not always be detected, and so if one is using only one dose, it may be erroneously concluded that CPCs are ineffective; however, such an error would be unlikely after three doses because the 13% increase in LVEF observed in the multiple-dose group (Fig. 4D) would be difficult to miss.

The present study was designed to provide robust results. Large numbers of rats (16–26) were included in each group. LV function and structure were assessed by a painstaking echocardiographic analysis and by another, independent method (hemodynamic studies), and both sets of measurements were conducted blindly. A thorough pathologic analysis was

performed, and survival of transplanted cells was assessed by three independent methods (PRC-based assays, Y-chromosome analyses, and mCherry/GFP immunohistochemistry).

As mentioned above, no previous investigation has used multiple doses of CPCs. The few studies that have compared multiple and single cell doses have used skeletal myoblasts[34, 35] and unfractionated bone marrow cells[32, 33, 36] and have yielded discrepant results (one of these studies examined only two treatments[32]). Yao et al.[32] found that two intracoronary infusions of autologous bone marrow mononuclear cells, 3 months apart, in patients with large MI resulted in further improvement of LVEF and decrease in scar size compared with one infusion. Premaratne *et al.*[35] reported that three repeated intramyocardial injections of skeletal myoblasts in rats starting 4 weeks after LAD ligation improved LV fractional area change (EF was not reported) and resulted in increased cell engraftment and LV elastance compared with a single injection. In contrast, Zhang *et al.*[33] reported no additive effects, and actually a trend toward worsening LV function, in mice receiving one, two, or three intramyocardial injections of bone marrow cells after coronary ligation. Our current findings that multiple doses have an additive effect on LV function are consistent with the observations of Gavira *et al.*[34]. These investigators injected one, two, or three doses of skeletal myoblasts transendocardially in pigs at 8, 14, and 20 weeks after MI; at 28 weeks after MI, animals that received three doses of cells exhibited a greater increase in LVEF vs. those that received a single dose.[34] Our study differs from these previous investigations in many respects, including (among other) the type of cell used, the animal model (reperfused, old MI), the methods to quantitate cell survival and fate, the measurement of cell proliferation, and the cell delivery technique (echo-guided intraventricular infusion).

Recently, Mann *et al.*[36] reported that a second injection of bone marrow mononuclear cells in previously responding patients with refractory angina produced an improvement in myocardial perfusion, anginal symptoms, and quality of life similar to that observed after the first injection performed ~ 5 years earlier. This study, however, did not compare multiple injections with one injection, nor did it demonstrate a cumulative effect.

Our PCR-based measurements of cell engraftment did not demonstrate a statistically significant difference between the single-dose and multiple-dose groups, both of which exhibited, on average, extremely low cell numbers (< 4,000 cells/heart). A considerable variability was observed, and it is possible that with greater sample sizes, a significantly greater content of CPCs may have been demonstrable in the multiple-dose group. Nevertheless, since each CPC infusion produced a short-lived "spike" of CPC concentration in the myocardium, the average tissue CPC concentration over time must have been greater in the multiple-dose vs. single-dose group, which would be expected to augment therapeutic efficacy.

The present study provides a new methodology for cell delivery that may have widespread application in preclinical research. All studies of CPCs performed to date have delivered cells via either the intramyocardial or the intracoronary route, both of which necessitate an open-chest procedure.[3–11] Performing multiple CPC injections with either of these approaches would be highly problematic, not only because of a host of technical and regulatory obstacles, but also because of the mortality associated with repeated

thoracotomies. These problems may be one of the reasons why repeated CPC administrations have never been investigated thus far. Our results demonstrate no myocardial retention of CPCs after i.v. infusion (Table 1).

To overcome these obstacles, we have developed a novel percutaneous delivery method that has enabled us to infuse CPCs into the LV cavity without opening the chest and without subjecting the animal to the stress of multiple surgeries. The closed-chest, echo-guided approach described herein has allowed us to successfully perform repeated injections with very low mortality: 68 of 73 rats survived after the 1st infusion, 66 of the 68 survived after the 2nd infusion, and 64 of the 66 survived after the 3rd infusion, for a total mortality of 12.3% (Supplementary Table I). Importantly, our PCR-based measurements of CPC retention at 24 h after infusion demonstrated that the number of cells present in the heart was similar to that found 24 h after intracoronary (open-chest) delivery (Table 1). The echo-guided percutaneous approach described herein was safe and well tolerated and can be repeated several times, potentially even more than three times. Therefore, our results introduce a new method that enables, for the first time, repeated cell administrations in rodents. This method is likely to be useful to investigators interested in maximizing the benefits of cell therapy. Repeated administration of cells is clinically relevant and eminently feasible in patients: once cells have been expanded, they can be frozen and stored for subsequent injection, which could be repeated at periodic intervals until the desired therapeutic effect is achieved, particularly in patients with ischemic cardiomyopathy, in whom the chronic nature of heart failure lends itself to repeated cell treatments.

What is the mechanism of the salubrious effects of repeated CPC administrations? Consistent with our previous studies,[4–7, 19] we found that, regardless of the number of treatments, very few transplanted CPCs engrafted and, of those, only a small fraction differentiated into mature cardiac myocytes, accounting for <1% of total myocytes (Fig. 9E). Consequently, the additive effects of repeated CPC infusions must be mediated by paracrine actions, just like those of a single CPC infusion. Among these actions could be decreased fibrosis in the noninfarcted region (Fig. 6D) and formation of new myocytes in the risk region, which is supported by the increase in BrdU[POS]/cTnI[POS] and IdU[POS]/cTnI[POS] cells (Fig. 8), the decrease in myocyte cross-sectional area (Fig. 7B), and the increase in myocyte density (Fig. 7C) in the risk region of the multiple-dose group. Also consistent with our previous studies,[4–7, 19] we found that CPC administration triggered a prolonged proliferative response in the risk region, the significance of which remains unclear. We have previously found that this phenomenon involves proliferation of endogenous CPCs, endothelial cells, and small cells expressing alpha-sarcomeric actin.[4–7, 19] Alternatively, or in addition, the BrdU[POS] and IdU[POS] cells may be newly-formed inflammatory cells originating in the bone marrow or spleen that infiltrate the risk region.

The present results have potentially far-reaching implications for the field of cell therapy. For example, in all clinical trials and almost all preclinical investigations conducted thus far, conclusions regarding whether a cell product was or was not effective have been based on the outcome of one cell administration. Our finding that the full effects of CPC administration require repeated treatments implies that the efficacy of cell therapy cannot be properly evaluated after a single dose. Just as it would not be appropriate to assess the

efficacy of an antibiotic on the basis of one dose, so it is not appropriate to assess the efficacy of a cell product on the basis of one treatment. If this new paradigm is applicable to other cell types and model systems, the conclusions of previous "negative" preclinical and clinical trials (which have used one dose of cells) could be questioned, and the protocols of future preclinical and clinical trials may have to be changed.

In conclusion, enthusiasm for cell therapy has been dampened by the modest or inconsistent improvement in LV function reported in many preclinical and clinical investigations. The present study identifies a possible reason for these results. We provide here the first demonstration that repeated CPC administrations are more effective than a single administration, and markedly so. In this rat model of chronic ischemic cardiomyopathy, each cell dose resulted in a similar increase in regional and global LV function, such that the cumulative improvement produced by three doses was roughly triple that produced by a single dose.

This concept may have major implications for both preclinical and clinical studies of cell therapy in general, because it implies that the protocols used heretofore (with a single dose of cells) may not have been adequate to test the efficacy of the treatment. In addition, we have described a new method that enables studies of repeated cell administrations in rodents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and Significance

What Is Known?

- All previous studies of c-kit[POS] cardiac progenitor cells (CPCs), and almost all preclinical and clinical studies of cell therapy in general, have assessed efficacy, or lack thereof, on the basis of one cell administration.

- The effects of one administration of CPCs (and of cell therapy in general) on left ventricular (LV) function have been regarded as modest or inconsistent.

- CPCs, and virtually all other types of cells tested heretofore, disappear quickly after transplantation, with minimal engraftment.

What New Information Does This Article Contribute?

- In rats with chronic ischemic cardiomyopathy (old myocardial infarction) that received three doses of CPCs 35 days apart, each cell dose resulted in a similar increase in regional and global LV function, such that the total cumulative improvement was roughly triple that produced by a single dose.

- Three CPC administrations were also associated with more viable tissue and less scar, less collagen in the risk and noninfarcted regions, and greater myocyte density in the risk region.

- Regardless of the number of treatments, very few transplanted CPCs engrafted and, of those, only a small fraction differentiated into mature cardiac myocytes, accounting for <1% of total myocytes.

- The present study provides a new method that enables, for the first time, repeated administrations of cells in rodents.

Although numerous cell types have been studied for the treatment of ischemic cardiomyopathy, enthusiasm for cell-based therapy has been dampened by the modest or inconsistent improvement in LV function reported in many preclinical and clinical investigations. The present study identifies a possible reason for these results. We provide here the first demonstration that repeated CPC administrations are more effective than a single administration, and markedly so. This concept may have major implications for both preclinical and clinical studies of cell therapy. For example, in all clinical trials and almost all preclinical investigations conducted thus far, conclusions regarding whether a cell product was effective or not have been predicated on the outcome of one cell administration. However, our finding that the full effects of CPC administration require repeated treatments implies that the efficacy of cell therapy cannot be properly evaluated after a single dose. This constitutes a veritable paradigm shift. If this new paradigm is applicable to other cell types and model systems, the conclusions of previous "negative" preclinical and clinical trials (which have used one dose of cells) may be questioned

(because a beneficial effect may have been missed), and the protocols of future preclinical and clinical trials may have to be changed to incorporate repeated treatments.
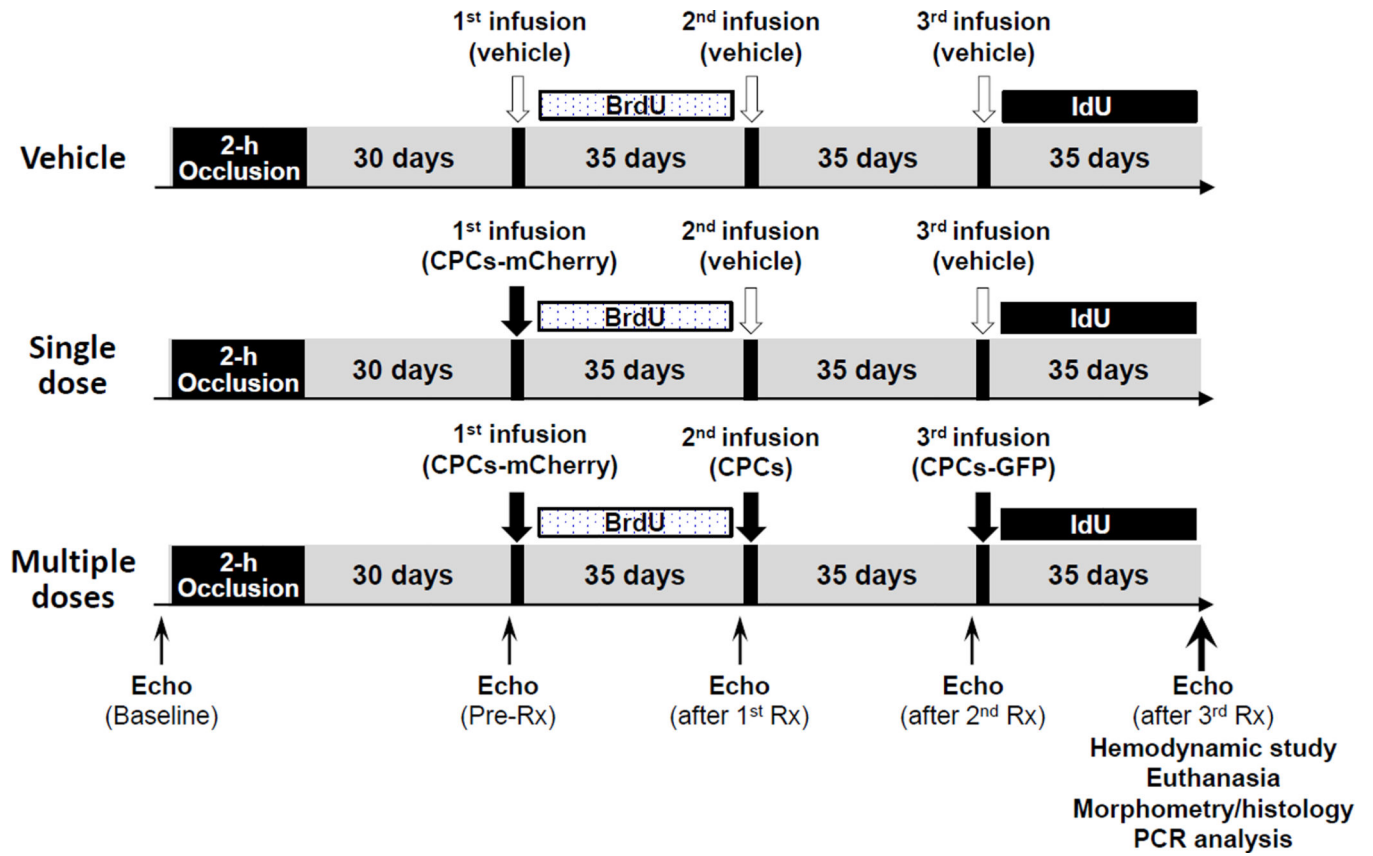
Figure 1. Experimental protocol
Echo, echocardiogram; CPCs-mCherry, mCherry-labeled CPCs; CPCs-GFP, GFP-labeled
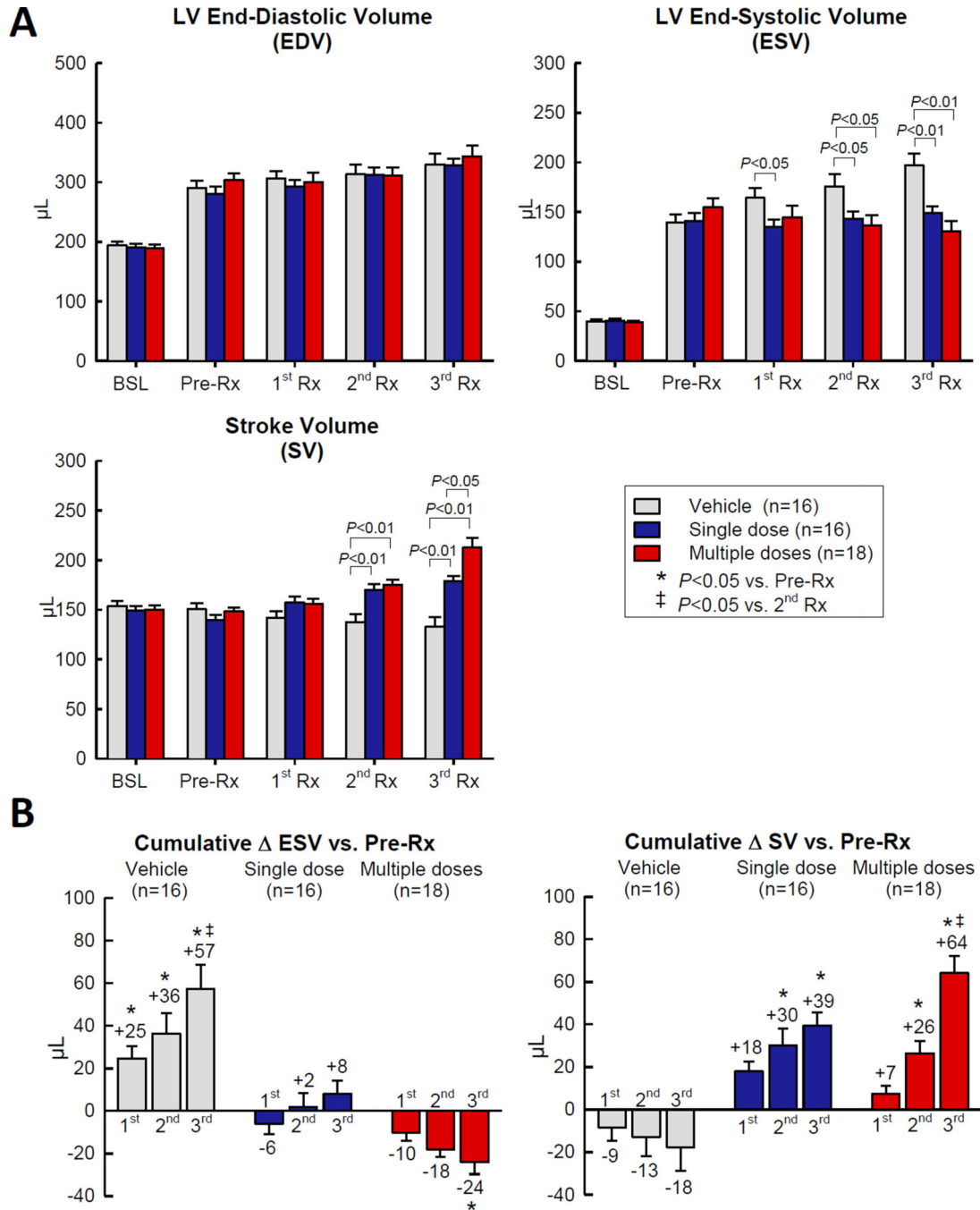CPCs; Pre-Rx, pretreatment (30 days after MI); 1st, 2nd, 3rd Rx: 1st, 2nd, and 3rd treatment.
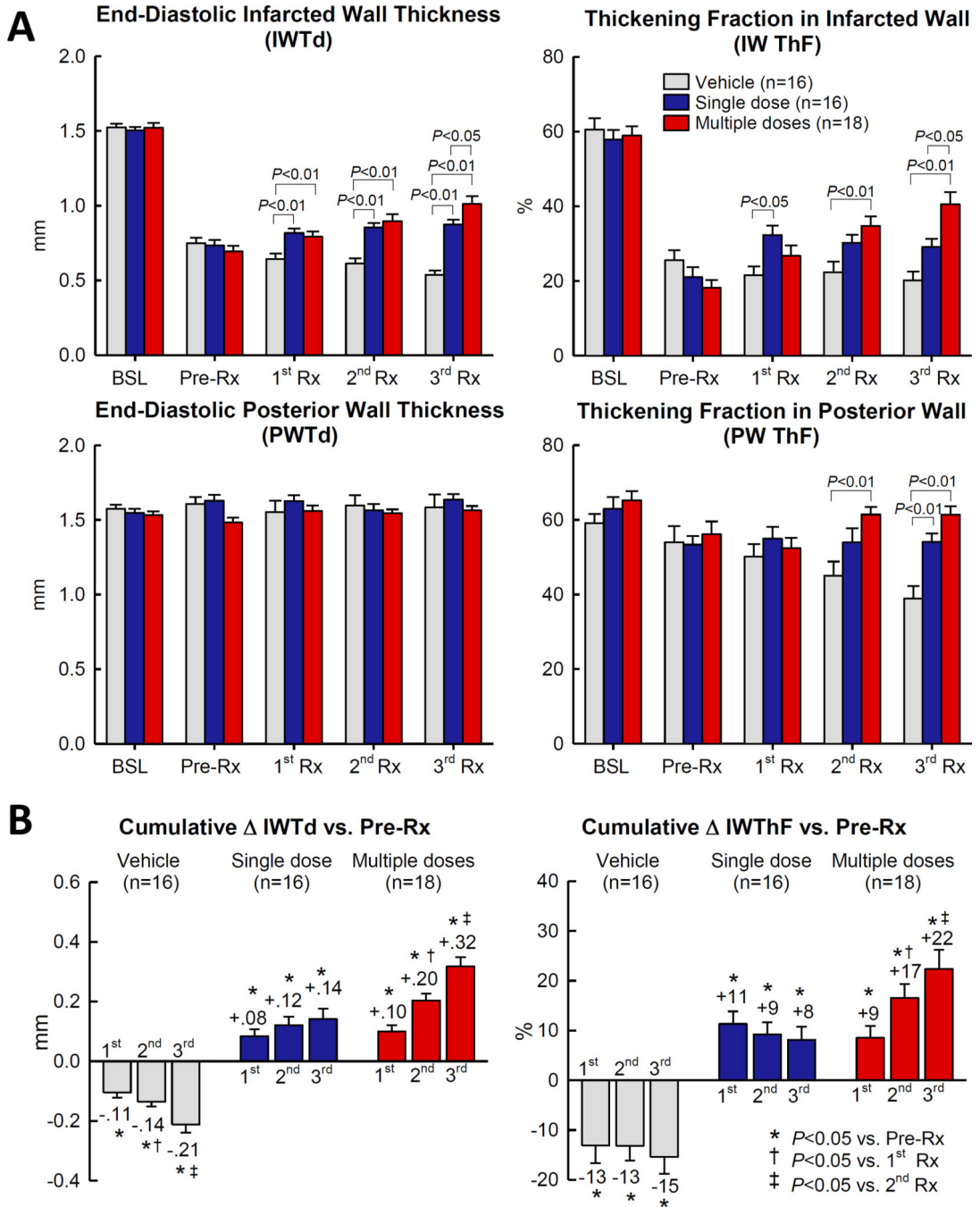
Figure 2. Echocardiographic assessment of LV volume

A. LV end-diastolic volume (EDV), end-systolic volume (ESV), and stroke volume (SV) in the vehicle, single-dose, and multiple-dose groups at baseline (BSL), before the 1st treatment (Pre-Rx) (i.e., 30 days after MI), 35 days after the 1st treatment (1st Rx), 35 days after 2nd treatment (2nd Rx), and 35 days after the 3rd treatment (3rd Rx); **B.** Cumulative changes in ESV (left) and SV (right) vs. pretreatment (Pre-Rx) values. Data are means ± SEM.
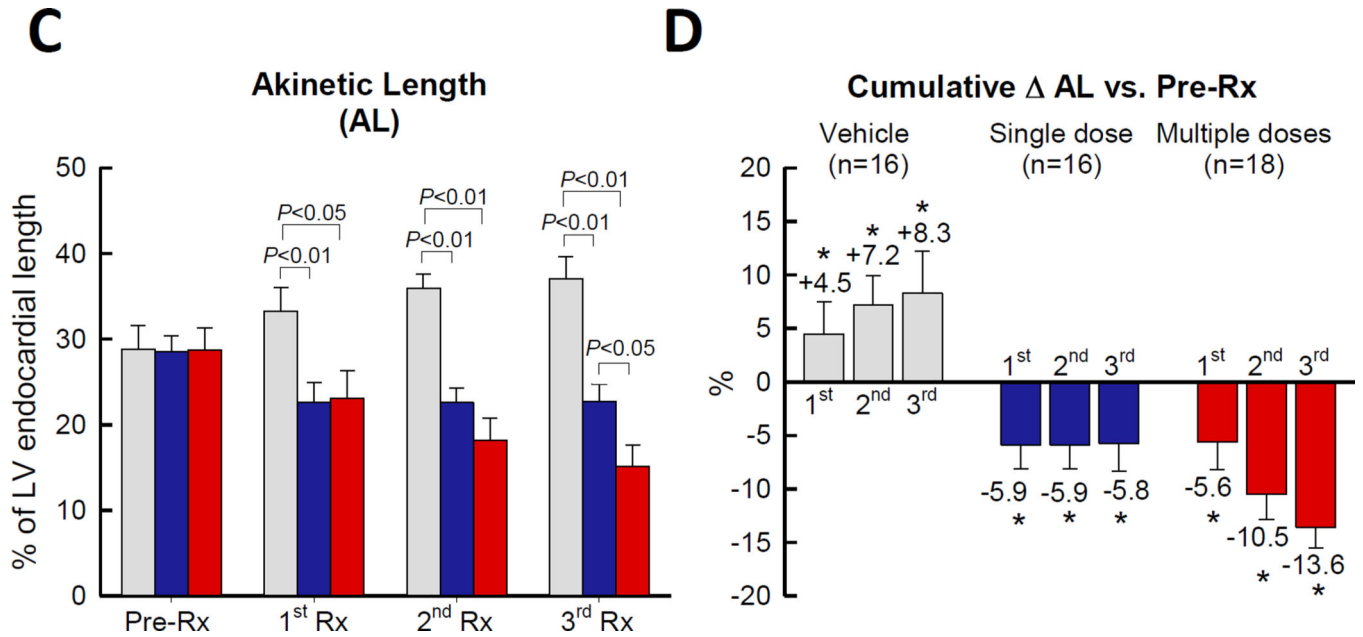
A

End-Diastolic Infarcted Wall Thickness (IWTd)

Thickening Fraction in Infarcted Wall (IW ThF)

Legend:
- Vehicle (n=16)
- Single dose (n=16)
- Multiple doses (n=18)

End-Diastolic Posterior Wall Thickness (PWTd)

Thickening Fraction in Posterior Wall (PW ThF)

B

Cumulative Δ IWTd vs. Pre-Rx

Cumulative Δ IWThF vs. Pre-Rx

* $P<0.05$ vs. Pre-Rx
† $P<0.05$ vs. 1st Rx
‡ $P<0.05$ vs. 2nd Rx

Figure 3. Echocardiographic assessment of regional LV function
A. End-diastolic thickness of the infarcted LV wall (IWTd), thickening fraction in the infarcted LV wall (IW ThF), end-diastolic thickness of the posterior (noninfarcted) LV wall (PWTd), and thickening fraction in the posterior (non-infarcted) wall (PW ThF) in the vehicle, single-dose, and multiple-dose groups at baseline (BSL), before the 1st treatment (Pre-Rx) (i.e., 30 days after MI), 35 days after the 1st treatment (1st Rx), 35 days after 2nd treatment (2nd Rx), and 35 days after the 3rd treatment (3rd Rx); **B.** Cumulative changes in IWTd and IW ThF vs. pretreatment (Pre-Rx) values. **C.** Akinetic endocardial length (akinetic length [AL]) before the 1st treatment (Pre-Rx) (i.e., 30 days after MI), 35 days after the 1st treatment (1st Rx), 35 days after 2nd treatment (2nd Rx), and 35 days after the 3rd treatment (3rd Rx); **D.** Cumulative changes in AL from pretreatment (Pre-Rx). Data are means ± SEM.
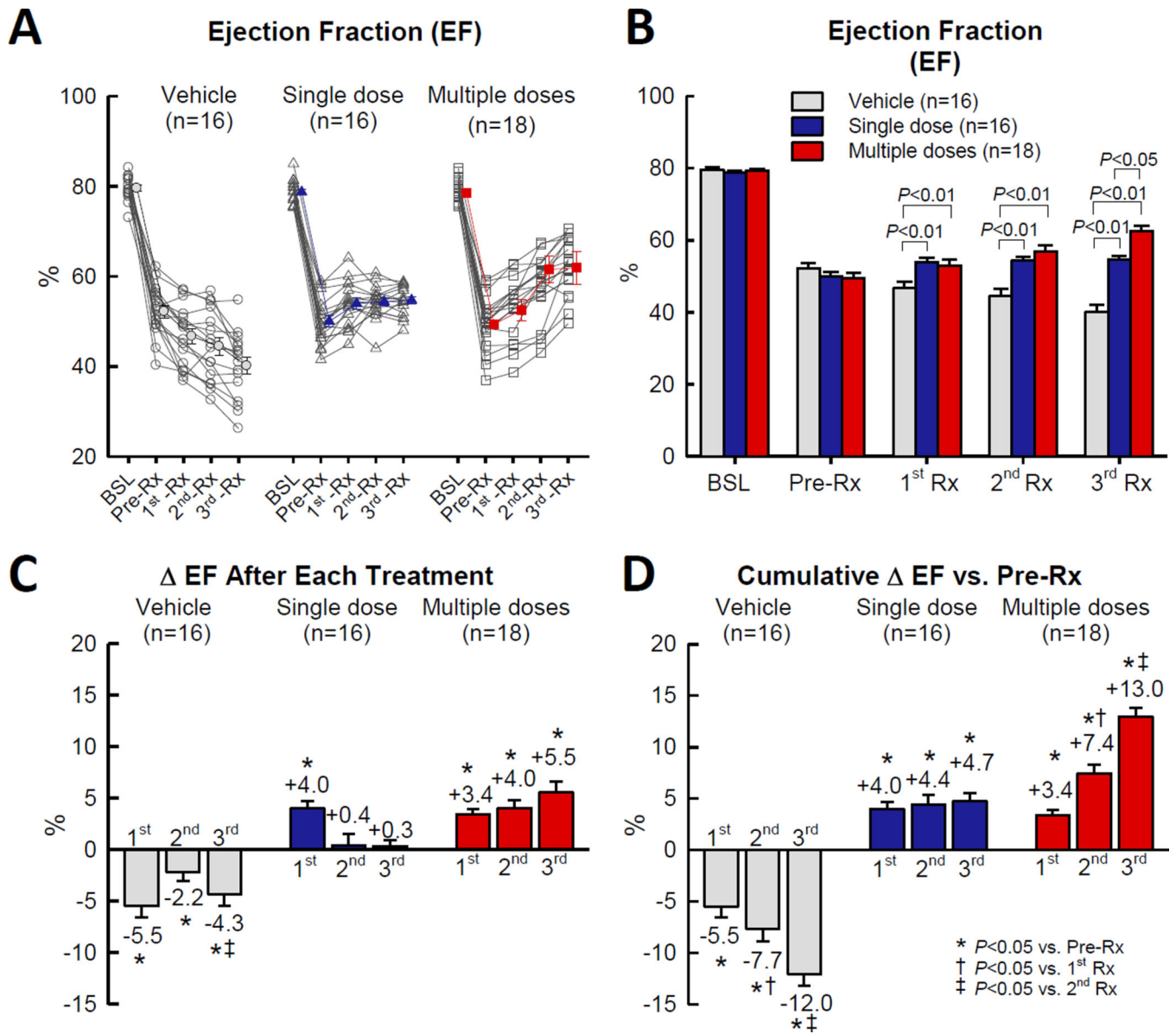
Figure 4. Echocardiographic assessment of global LV function
A. Line plots showing the time-course of LV EF in individual animals (black lines) and average values of LV EF in the vehicle, single-dose, and multiple-dose groups (colored lines); **B.** Bar graph illustrating LV EF at baseline (BSL), before the 1st treatment (Pre-Rx) (i.e., 30 days after MI), 35 days after the 1st treatment (1st Rx), 35 days after 2nd treatment (2nd Rx), and 35 days after the 3rd treatment (3rd Rx); **C.** Changes in LV EF (absolute units) at 35 days after the 1st, 2nd, and 3rd treatment vs. the respective pretreatment values; **D.** Cumulative changes in LV EF (absolute units) vs. pretreatment (Pre-Rx) values. Data are means ± SEM.
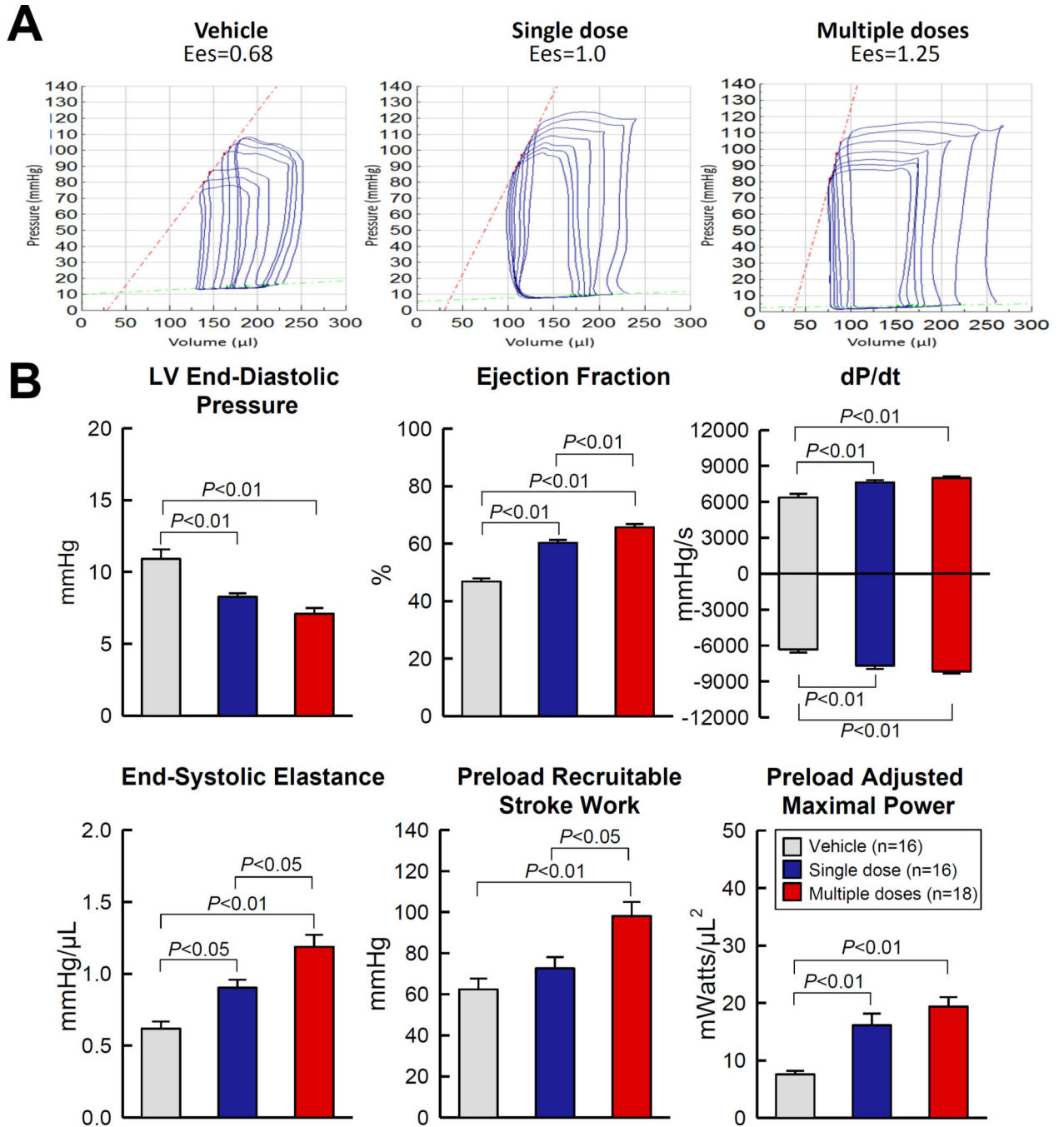
Figure 5. Hemodynamic assessment of LV function

Hemodynamic studies were performed with a Millar conductance catheter at 35 days after the 3rd treatment, just before euthanasia. **A.** Representative pressure-volume loops recorded during preload manipulation by brief inferior vena cava occlusions. **B.** Quantitative analysis of hemodynamic variables. Data are means ± SEM.
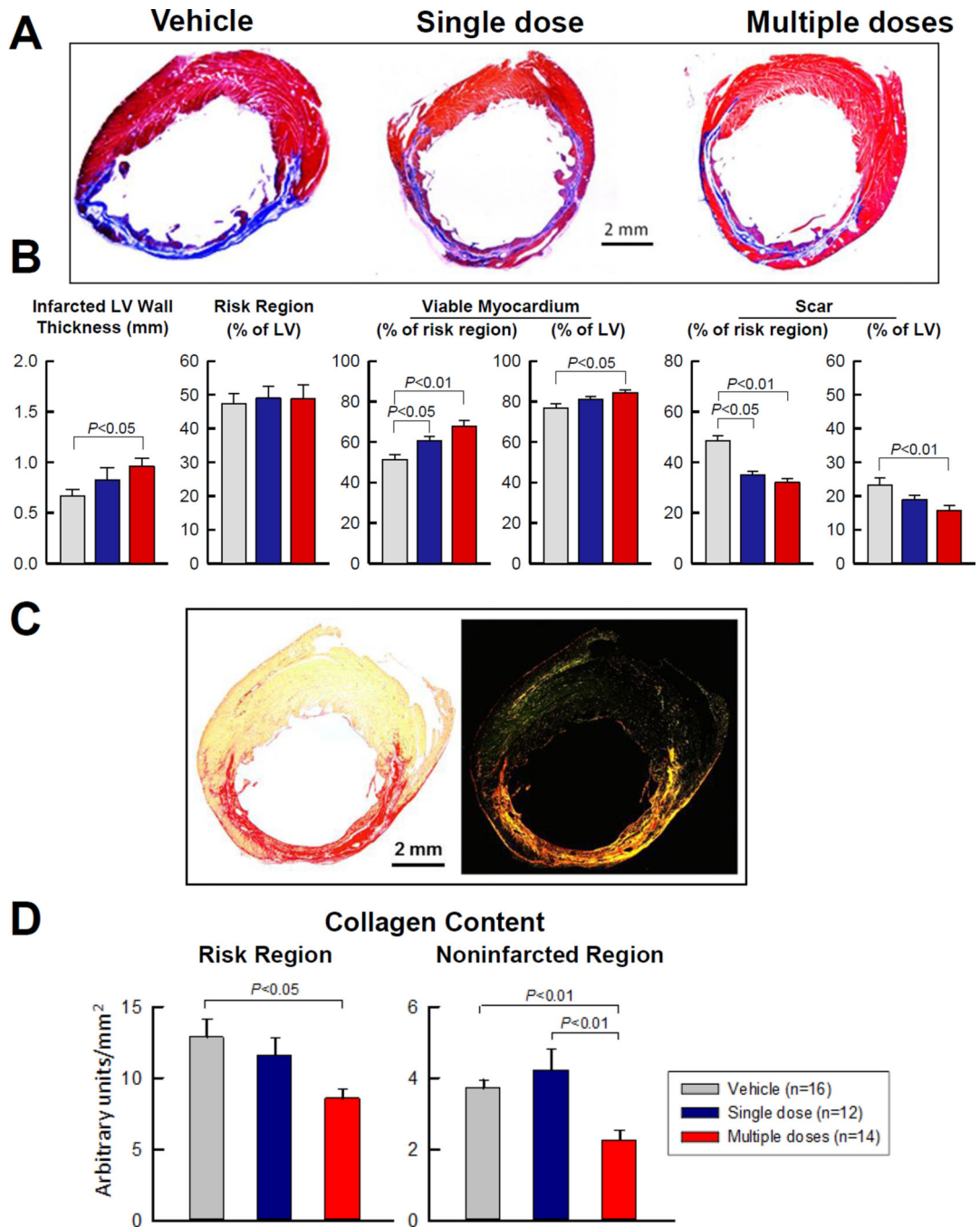
Figure 6. Morphometric analysis (A and B) and myocardial collagen content (C and D)
A. Representative Masson trichrome-stained myocardial sections. Scar tissue and viable myocardium are identified in blue and red, respectively. **B.** Quantitative analysis of LV morphometric parameters. The size of the risk region, left ventricle (LV), viable myocardium, and scar was calculated in mg. The risk region comprises both the border zones and the scarred region. **C.** Representative microscopic images of an LV section stained with picrosirius red; images were acquired with transmission light (left) or polarized light

(right). **D.** Quantitative analysis of polarized light microscopic images showing collagen content per mm^2 in the risk and noninfarcted regions. Data are means ± SEM. Bar is 2 mm.
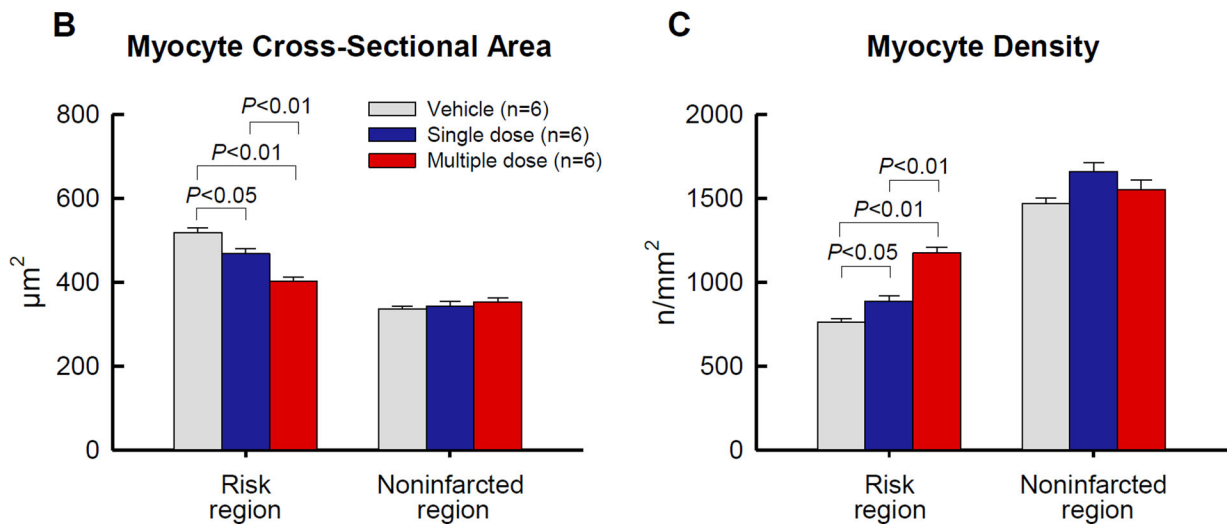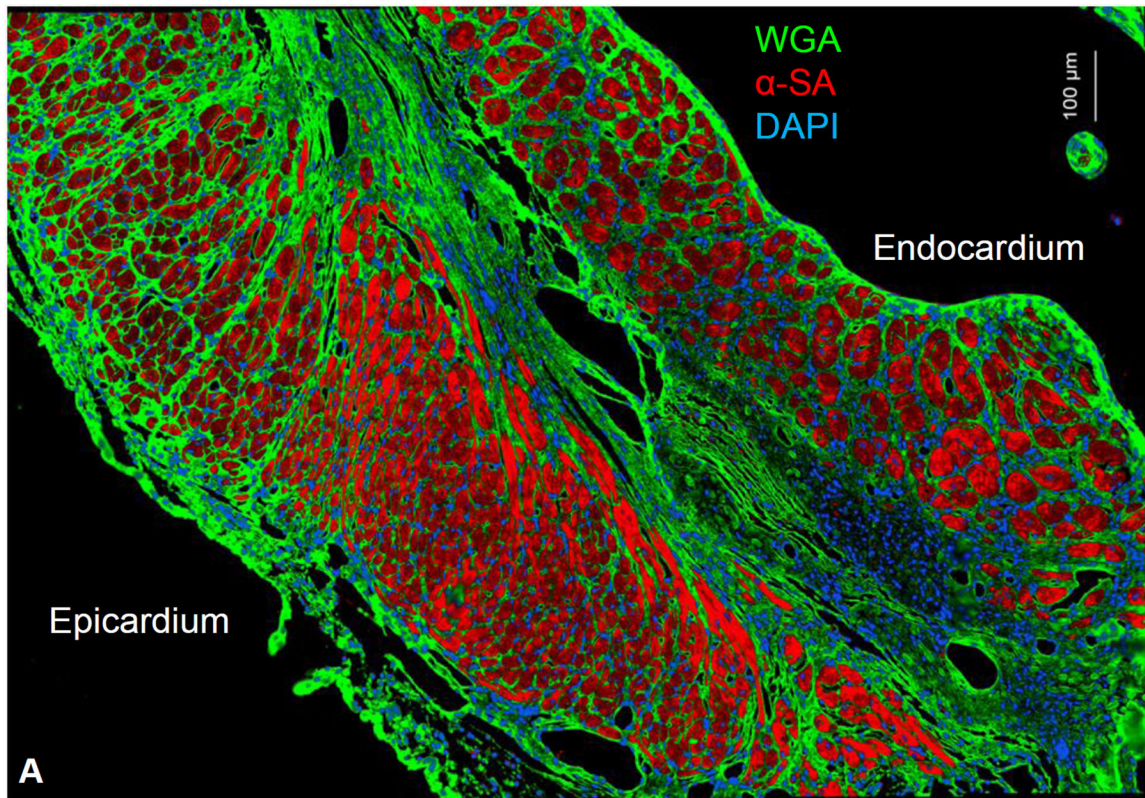
Figure 7. Analysis of myocyte cross-sectional area and myocyte density
Myocyte cross-sectional area and myocyte density were determined in rat hearts stained with WGA (green) and α-SA (red). **A.** Representative epifluorescent microscopic image, sequentially acquired from 18 fields of the infarcted region of a vehicle-treated rat at a magnification of x300. Myocytes with round nuclei and clearly defined sarcolemmal borders were selected for analysis of cross-section area. **B and C.** Quantitative analyses of myocyte cross-sectional area (**B**) and myocyte density (**C**) per mm^2. WGA binds to the myocyte membrane, thereby facilitating the evaluation of myocyte cross-sectional area and density.
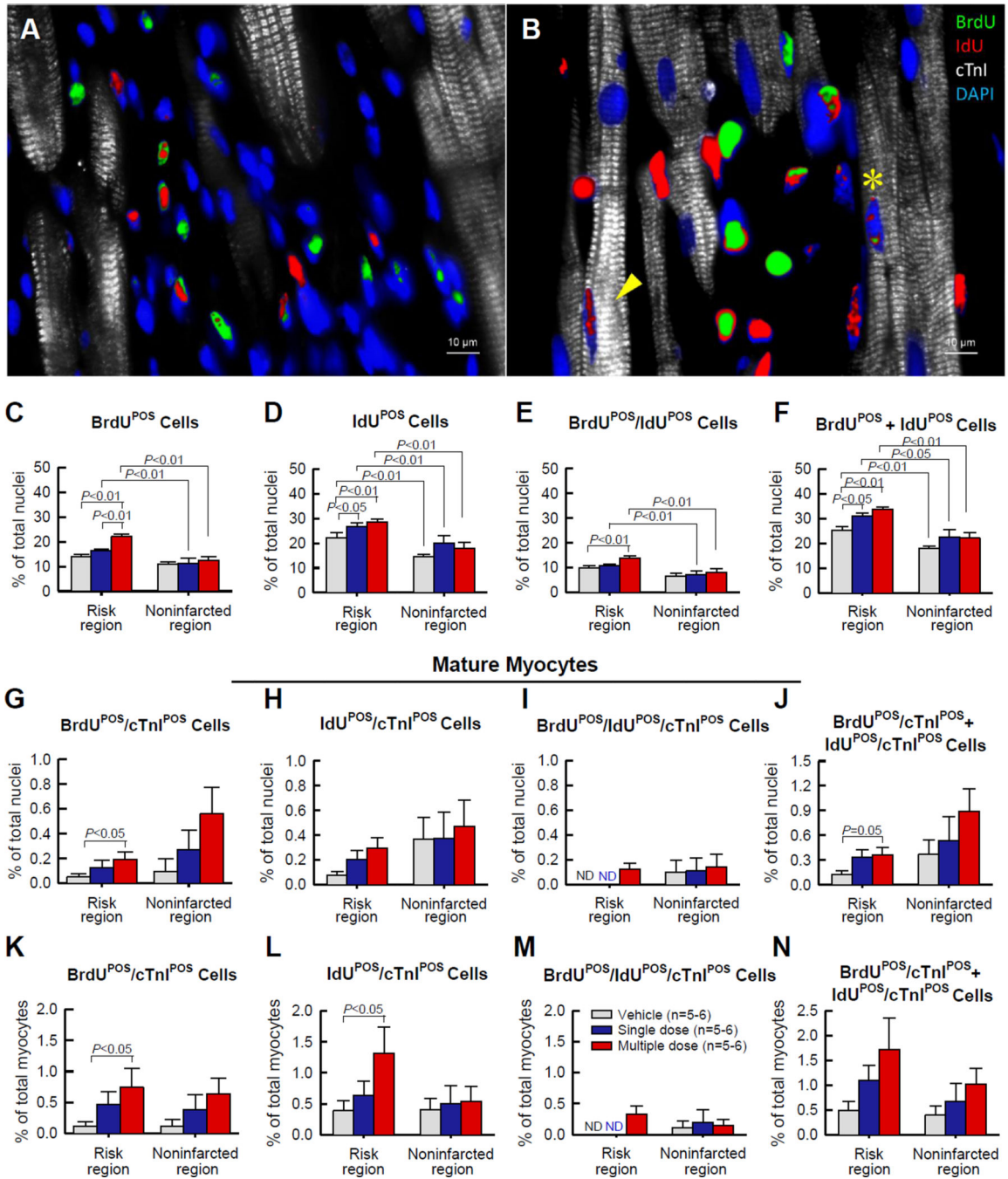
The risk region comprises both the border zones and the infarcted region. Data are means ± SEM. Bar is 100 μm.

Figure 8. Proliferation of CPCs and mature myocytes

Rats received BrdU infusion for 35 days after the 1st CPC administration and IdU infusion for 35 days after the 3rd CPC administration. **A and B.** Representative confocal microscopic images acquired from the infarcted region (**A**) and the border zone (**B**) of a vehicle-treated rat. Positivity for BrdU is shown in green and for IdU in red. Myocytes were stained with an anti-cTnI antibody (white) and nuclei with DAPI (blue). In **B**, the yellow arrowhead indicates an IdU^{POS} mature myocyte, whereas the yellow asterisk shows a BrdU^{POS}/IdU^{POS} mature myocyte. **C–N.** Quantitative analysis of the number of BrdU^{POS}, IdU^{POS}, and

BrdU/IdU^{POS} cells expressed as a percent of total nuclei (**C–F**) and the number of BrdU^{POS}, IdU^{POS}, and BrdU/IdU^{POS} myoctes expressed as a percent of total nuclei (**G–J**) and as a percent of total myocytes (**K–N**). The region at risk comprises both the border zones and the scarred region. Data are means ± SEM. Bar is 10 μm.
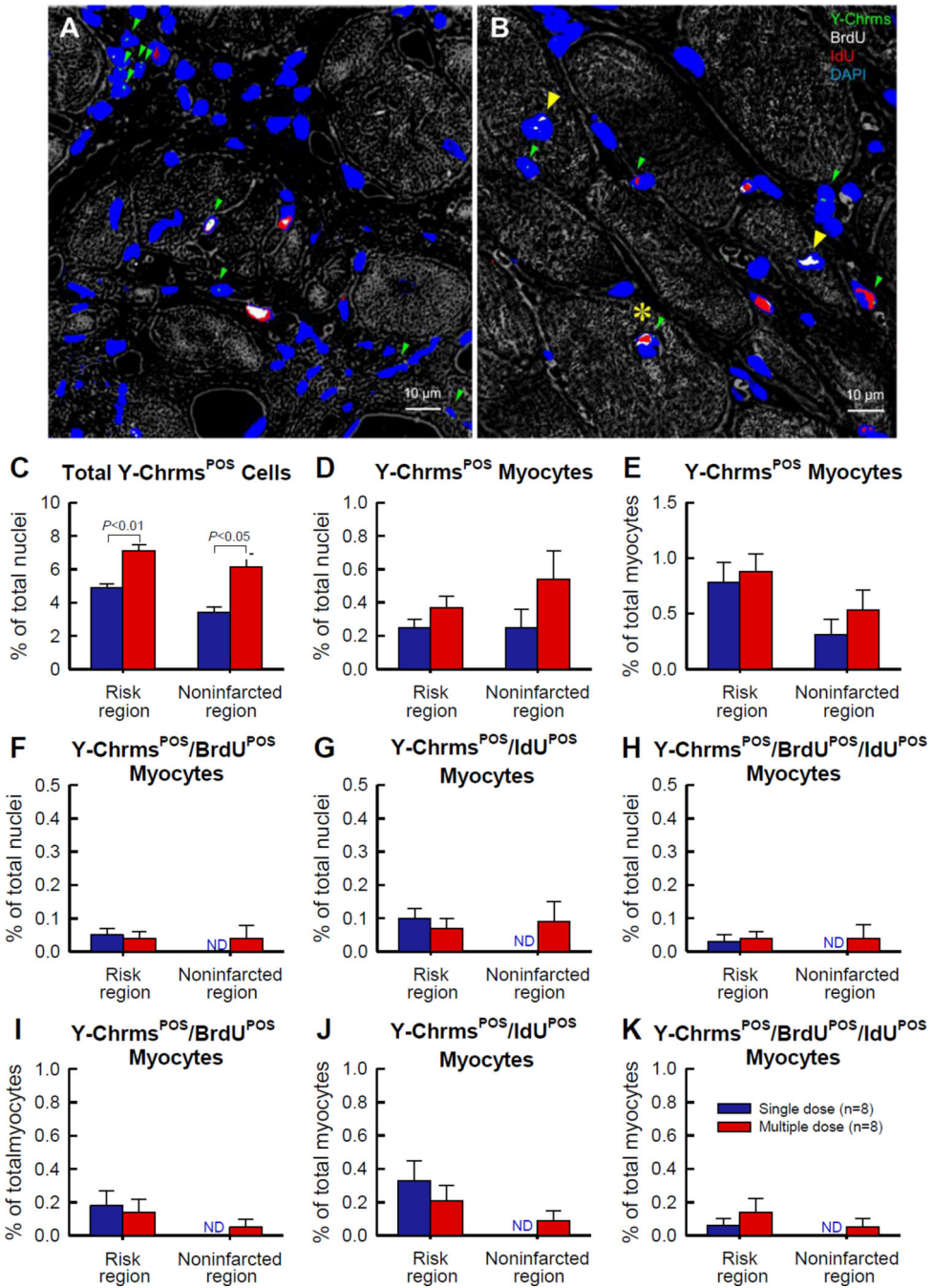
Figure 9. Analysis of Y-Chromosome^{POS} cells

A–B. Representative confocal microscopic images acquired from the infarcted region (**A**) and border zone (**B**). Green arrowheads indicate Y-chromosome fluorescent signals (green/cyan) in nuclei. Note a cluster of five Y-chromosome^{POS} nuclei in A (top left), suggesting Y-chromosome^{POS} cell division. Yellow arrowheads indicate BrdU^{POS} mature myocytes, whereas the yellow asterisk shows a Y-chromosome^{POS}/BrdU^{POS}/IdU^{POS} mature myocyte (**B**). BrdU is shown in white, IdU in red, and nuclei are stained with DAPI in blue. Myocardial morphology was examined with the confocal transmitted light channel's

detector (ChD) in which the pseudocolor selected for the myocardial background in the ChD channel was gray white. **C–K**. Quantitative analysis of the number of Y-chromosome^{POS}, BrdU^{POS}, and IdU^{POS} cells. The risk region comprises both the border zones and the infarcted region. Data are means ± SEM. Bar is 10 μm.

Table 1

Pilot Studies to determine myocardial CPC retention

Route of CPC Delivery	n	CPC Number Delivered	CPC Number Retained in Heart 24 h After Delivery
Intracoronary infusion	3	1.0×10^6	$118{,}924 \pm 24{,}458$
Intravenous infusion	4	3.0×10^6	Not detectable
Echo-guided intraventricular injection	3	3.0×10^6	$24{,}680 \pm 12{,}570$
	3	9.0×10^6	$49{,}580 \pm 4{,}982$
	6	12.0×10^6	$112{,}983 \pm 56{,}300$

Data are means ± SEM.

Table 2

Persistence of transplanted CPCs in myocardium

n=14 hearts	Risk Region	Noninfarcted Region
mCherry^{POS} cells (cells/mm^2)	0.02±0.01	0
GFP^{POS} cells (cells/mm^2)	1.50±0.20	0.58±0.20

Data are means ± SEM. These rats received intraventricularly 12 million mCherry-labeled CPCs during the 1st infusion and 12 million GFP-labeled CPCs during the 3rd infusion.