Effect of Cardiac Stem Cells on Left Ventricular Remodeling in a Canine Model of Chronic Myocardial Infarction

Welt et al: Cardiac Stem Cells in Chronic Myocardial Infarction

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Abstract

Background—Regenerative medicine including cell therapy is a promising strategy for recovery of the damaged myocardium. C-kit-positive cardiac stem cells (CSCs) have been shown to improve myocardial function after ischemic injury in animal models and in early clinical experience. We used a chronic large animal model of myocardial infarction (MI) with substantial reductions in left ventricular ejection fraction (LVEF) and adverse remodeling to examine the effect of late autologous CSC intramyocardial injection on long term cardiac structure and function.

Methods and Results—Thoracotomy and ligation of the proximal LAD, additional diagonal branches, and atrial biopsy for CSC culture was performed in canines. Baseline cardiac MRI was performed at 6 weeks post-infarct followed by repeat thoracotomy for randomization to intramyocardial injection of CSCs (n=13) or vehicle alone (n=6). At 30 weeks post MI, repeat MRI was performed. Data was analyzed using nonparametric tests (Wilcoxon signed rank and rank sum tests). In control animals, left ventricular end systolic volume (LVESV) and end-diastolic volume (LVEDV) increased from 6 to 30 weeks (Median and interquartile range (IQR) 51.3 ml (43.3, 57.4) to 76.1 ml (72.0, 82.4), p=0.03 and 78.5 ml (69.7, 86.1) to 99.2 ml (97.1, 100.4), p=0.03). LVEF declined further (35.2% (27.9, 38.7) to 26.4% (22.0, 31.0), p=0.12). In the cell treated animals, this late adverse LV remodeling was attenuated (LVESV42.6 ml (38.5, 50.5) to 56.1 ml (50.3, 63.0), p=0.01 vs. control). There was a non-significant attenuation in the increase in LVEDV (64.8 ml (60.7, 71.3) to 83.5 ml (74.7, 90.8), p=0.14 vs. control) and LVEF change over time differed (30.5% (28.4, 33.4) to 32.9% (28.6, 36.9), p=0.04 vs. control).

Conclusions—Intramyocardial injection of autologous CSCs in a late phase model of chronic infarction resulted in less increase in LVESV and preservation of LVEF.

Key Words: chronic heart failure; remodeling heart failure; stem cells; animal model
Regeneration and repair of myocardium through cell therapy offers the potential for recovery of function after ischemic injury in both the acute and chronic setting. Several unipotent and multipotent cell types including human embryonic stem cells\(^1\), c-kit-positive (c-kit\(^{\text{pos}}\)) hematopoietic stem cells\(^2\), CD34-positive cells\(^3\), side population bone marrow cells\(^4\), bone marrow mesenchymal stem cells\(^5\), bone marrow mononuclear cells\(^6\), and resident cardiac progenitor cells\(^7\)\(^8\) have demonstrated some cardiac regenerative capacity in animal models.

Despite multiple studies in both animals and humans, many questions remain including what is the appropriate cell type, the best method of delivery, the correct timing of administration, and the mechanism and extent of functional improvement. Since the use of xenogenic cells in the majority of these studies limits the duration of observation, the durability of the effect has not received the same attention. This study was designed to utilize a canine model of chronic infarction with progressive adverse remodeling to examine the effects of intra-myocardial autologous cardiac stem cell (CSC) injection on the late phase of adverse LV remodeling.

CSCs defined as c-kit\(^{\text{pos}}\) cells isolated from cardiac tissue have been demonstrated to be self-renewing, clonogenic, and multipotent both in-vitro and in-vivo\(^8\). CSCs have been shown to alleviate myocardial dysfunction in multiple animal studies when delivered following acute myocardial infarction. These cells have been demonstrated to form new functional and contracting myocardium as well as to form new capillaries, arterioles, and large arteries\(^9\). Decreased infarct size, less chamber dilation, and improved hemodynamic conditions have been reported\(^10\). Two categories of CSCs have been identified. CSCs with predominantly myogenic potential are termed mCSCs. In addition, a subset of human CSCs identified by the cell marker KDR termed vascular progenitor cells (vCSCs) have been shown to form new conductive
arteries leading to improved coronary flow and improved ventricular function when injected into immune-suppressed canines at the site of coronary vascular constriction\textsuperscript{11}.

We addressed the question of whether intra-myocardial injection of CSCs into the chronically infarcted heart would lead to beneficial effects on the late phase of adverse cardiac remodeling. In order to do this we utilized autologous cells cultured from cardiac tissue collected at the time of experimental myocardial infarct and then delivered 6 weeks later. We followed the animals for 30 weeks to address the hypothesis that CSCs would favorably alter the late phase of adverse LV remodeling post MI. In addition, we asked a second question of whether injection of mCSCs in the body of the infarct and vCSCs at the site of coronary ligation would lead to an enhanced recovery of function.

Methods

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (Eighth Edition, Revised 2010). Our experimental design (Figure 1) consisted of infarct creation, followed by MRI and cell injection at 6 weeks and randomization to one of three groups (control, mCSC injection within the body of the infarct and at the LAD ligation site, and mCSC injection in the body of the infarct with vCSCs given at the LAD ligation site). Animals were allowed to recover for 24 weeks and then re-imaged and sacrificed.

Infarct creation: Male mongrel dogs aged 7 to 18 months and weighing 17 to 25 kg, underwent myocardial infarct creation by open surgical technique with suture ligation of the left anterior descending artery (LAD) and diagonal branches under direct visualization. Induction
and maintenance anesthesia were accomplished with Propofol (5 mg/kg) and isoflurane (1-4%).
A left, muscle-sparing thoracotomy provided direct access to the LAD which was ligated proximally beyond the first septal artery, distally, and at its diagonal branches with non-absorbable suture (3-O Ethibond, Ethicon, Somerville, NJ, USA). For arrhythmia prophylaxis, continuous intravenous lidocaine (40-100 mcg/kg/min) was provided. Effectiveness of ligation was confirmed by angiography performed via transfemoral arterial access. The thoracotomy was closed initially with heavy vicryl sutures to achieve rib re-approximation. Then, a three layer subcutaneous closure with vicryl sutures was used. A chest tube was used to evacuate the pleural space and re-establish negative intra-thoracic pressure and removed at the end of the procedure.

A fentanyl transdermal patch (75 mcg/hr) and oral meloxicam (0.1 mg/kg/day) provided postoperative anesthesia. Troponin I was measured 12 to 24 hours post-procedure.

Cell isolation and culture: During the initial surgery, a sample of the left atrial appendage was collected, finely divided (<1 mm³) and suspended in 15 ml Ham’s F12 medium containing 1-3 mg/ml of collagenase NB 4. As previously described and as used in the recent SCIPIO clinical trial, tissue samples were enzymatically dissociated to obtain single cell suspensions. Following digestion, cells were plated in culture dishes containing Ham’s F12 medium supplemented with 10% fetal bovine serum, 100 ng/ml recombinant human bFGF, 0.2 mM L-glutathione, and 5 mU/ml human erythropoietin (Sigma). Subsequently, cells were expanded and subjected to immunomagnetic sorting with rabbit anti-c-kit (Santa Cruz Biotechnology) and magnetic microbeads (CD117 MicroBead kit, Miltenyi) to obtain c-kit-positive CSCs. C-kit positive cells were further analyzed and sorted by FACS according to the expression of KDR (KDR Antibody, Abcam Ab2349). C-kit-positive cells and cells positive for c-kit and KDR corresponded to mCSCs and vCSCs, respectively. Importantly, both classes of CSCs were
obtained in all animals studied. After enrichment, mCSCs and vCSCs were expanded in F12K medium supplemented with 10% fetal bovine serum (FBS). mCSCs were expanded until passage 7-8 (P7-P8). Additionally, c-kit and c-kit-KDR positive CSCs (vCSCs) were analyzed by flow-cytometry for the evaluation of their undifferentiated state. Cellular debris and aggregates were gated out based on forward and side scatter. Gating on the signal of the nuclear stain DAPI was employed to exclude additional artifacts. Isotype-matched negative controls were utilized to define the threshold for each specific signal and establish the appropriate gate for positive cells. Data were analyzed with the instrument software. FACS analysis was performed with FACS Aria (Becton Dickinson) or Accuri C6 (Accuri Cytometers) instruments. Approximately 10 x 10^6 CSCs were obtained from each dog.

**Cardiac MRI (CMR):** Animals were anesthetized via the same protocol for surgery and were studied supine in a 3.0T CMR system (Signa HDx, GE Healthcare, USA) with an 8-element phased-array surface coil. CMR study consisted of cine steady state free precession imaging (typical TR/TE 3.4/1.2ms, in-plane spatial resolution 1.3x1.5mm) of LV function and late gadolinium enhancement (LGE) imaging (TR/TE 4.8/1.3ms, TI 200-300ms) for myocardial scar. All images were acquired using ECG gating and temporary cessation of mechanical ventilation to minimize cardiac and respiratory motion, respectively. Cine and LGE imaging were obtained in 8-14 matching short-axis (6 mm thick with 0 mm spacing) and 3 radial long-axis planes. A previously described segmented inversion-recovery pulse sequence for LGE imaging was used starting at 15 minutes after a cumulative 0.15mmol/Kg dose of gadolinium-DPTA (Gadopentetate Dimeglumine, Bayer HealthCare, Wayne, NJ).

All images were analyzed with specialized software (QMass®, Medis, Leiden, The Netherlands). All LGE images were analyzed at a separate session from the interpretation of
segmental wall motion. We interpreted LGE as present or absent and quantified the myocardial mass of the LGE by a semi-automatic detection method. We followed the 17-segment nomenclature in assigning the coronary distribution of the myocardial segments with abnormal LGE\(^\text{15}\). To quantify the myocardial mass of the LGE, the endocardial and epicardial borders of the short-axis LV on LGE imaging were manually traced. Then the computer-assisted detection algorithm defined LGE as any region with a signal intensity >2 SD above a reference remote myocardial region as previously reported\(^\text{16}\). We expressed LGE mass as both grams of tissue and as well as a percentage of the LV mass (LGE\%). We manually traced epicardial and endocardial borders of matching short-axis cine locations at end systole and end-diastole to determine the LV ejection fraction (LVEF), end-diastolic volume (LVEDV), end-systolic volume (LVESV), and the LV myocardial mass (end-diastole only)\(^\text{17,18}\). LVEF was measured by standard Simpson’s Rule\(^\text{18}\).

Cell injection: Only animals with a measured infarct size of greater than 5% of LV mass were continued in the study. One to 2 days post MRI, the animals were sedated and intubated and underwent a second thoracotomy. angiography was performed to verify the site of LAD ligation to allow for identification of sites for injection. The day of the injections, mCSCs and VCSCs were exposed for 2 h to IGF-1 and HGF. After the stimulation with growth factors, mCSCs were detached and counted to obtain a single cell solution with a concentration of 1 x 10\(^6\) cells/ml: 0.8 ml was loaded in each syringe. According to a pre-specified block randomization scheme (1:1:1 for 3 groups; control, mCSCs, and mCSCs with vCSCs), cells were delivered in 1 ml syringes labeled for the proximal site and distal site. According to the protocol, 8 injections (8 x 0.1 ml = 8 x 100,000 cells) were performed in each of the two sites of delivery. Operators were blinded to the content of the syringes. Purse string sutures (3-0 Ethibond) were
placed around two needle entry sites, one at the proximal LAD ligation sites and then one further down the LAD within the body of the infarct. Utilizing a 25 gauge needle bent by hand at the operating table to approximately 45 degrees, the cells were delivered into the myocardium in parallel with the anterior wall without entering the ventricular cavity (Figure 2). Aliquots of cells, or control medium were delivered in a radial pattern from roughly a 1 o’clock to a 5 o’clock position via separate 0.1 to 0.2 ml injections along each radial line. For each new radius, the needle was redirected without withdrawing the tip from the myocardium. As per the experimental design, groups were designed to include PBS injections at proximal and a distal site, mCSCs at both sites, and vCSCs in the proximal site and mCSCs in the distal site. After completion of delivery, each purse-string suture was closed over the entry site during withdrawal of the needle to limit the escape of cells. The thoracotomy was closed as described above and the animals were allowed to recover with the same pain-monitoring and control procedures.

Terminal studies: At 30 weeks post MI, the animals underwent repeat MRI. One to 2 days later, they were sedated and placed under general anesthesia. Femoral access was obtained and the animals underwent angiography to document the state of the coronary anatomy. The aortic valve was crossed and baseline LV systolic and end-diastolic pressures were measured. Afterwards, the chest was opened, 20,000 units of heparin were given intravenously, and all vessels originating from the aortic arch were ligated. Euthanasia solution (sodium pentobarbital 390 mg/ml and sodium phenytoin 50 mg/ml) was given at 150 mg/kg and then the heart was arrested in diastole by the injection of potassium chloride, 100 mM, into the left ventricle. The descending aorta was ligated and cannulated, the heart perfused retrograde with normal saline and then with 10% buffered formalin. The perfusion pressure was maintained at 100 mmHg. The
heart was then removed and dissected. The areas of injection were identified by the presence of suture and marked for histologic location.

Histology: After photographing, hearts were differentially colored over the anterior surface to grossly identify regions of prior injections. Hearts were then serially transversely sectioned at approximately 1 cm intervals from apex to base (6-7 slices), photographed, and the volumes of associated scar estimated (Figure 3). Histology sections were sampled from the basal face of each slice, with sections bridging both injected and non-injected portions of the specimen. Routine hematoxylin and eosin staining, as well as Masson’s trichrome staining was performed on 10 μ thick sections.

Immunohistochemistry was performed on 5-10 μ-thick formalin-fixed, paraffin-embedded tissue sections using anti-human specificity antibodies unless otherwise indicated; internal control staining demonstrated adequate cross-reactivity of the canine tissues (see supplemental material for specifics). Tissues were analyzed in a blinded fashion, without knowledge of the nature of the injected cells or whether recipient hearts received control saline only.

Statistics: Results are expressed using the median and interquartile range (IQR). The primary planned comparison in the study was LVESV change in the control vs. combined cell-treated group with secondary analyses of other MRI parameters. Intragroup analyses (control and treated 6 week vs. 30 week) were analyzed with Wilcoxon signed-rank tests. For the primary intergroup comparisons (control vs. treated and mCSC vs. mCSV/vCSC, change over time), data were analyzed by comparing the changes from baseline using the Wilcoxon rank sum test utilizing STATA data analysis and statistical software. Similar comparisons were performed within the cell treated groups comparing mCSC vs. mCSC/vCSC groups.
Results

Twenty eight animals underwent initial myocardial infarction (Figure 1). Three animals with a 6 week MI size < 5% were excluded prior to randomization. Five animals died prior to cell injection (2 during the acute infarction and three died at post-op days 15, 16, and 18 respectively without apparent prior symptoms). Nineteen of the 20 randomized animals completed the protocol through the cell injection and terminal hemodynamics and MRI. One animal (mCSC group, initial infarct size of 14.7%, EF 25%) died suddenly at post-operative day 11.

Control group: At 6 weeks post infarct, LVEF was markedly reduced and the ventricular volumes increased from normal canine values\textsuperscript{19-22} (Table). In the control group, there was progressive further dilation of the ventricle from 6 weeks to 30 weeks with increases in LVESV (Median (IQR) 51.3 (43.3, 57.4) ml to 76.1 (72.0, 82.4) ml, p=0.03, Figure 4) and LVEDV (78.5 ml (69.7, 86.1) to 99.2 (97.1, 100.4)ml, p=0.03, Figure 5). LVEF at 6 weeks post infarct was reduced at 35.2% (27.9, 38.7)and over the ensuing 24 weeks fell to 26.4% (22.0, 31.0) (p=0.12, Figure 6). Infarct size as a percent of LV mass fell from 11.7% (8.6, 17.4) at 6 weeks to 9.7% (5.0, 11.2)at 30 weeks (p=0.03). Infarct volume fell from 6.2 (4.2, 7.6) at 6 weeks to 4.8 ml (4.6, 7.7) at 30 weeks (p=0.13).

CSC therapy: The 6 week post infarct values for LVEF and chamber volumes for the cell treated animals were similar to the control group (Table). However, in the cell treated animals, the increase in LVESV was attenuated (42.6 ml (38.5, 50.5) to 56.1 ml (50.3, 63.0), p=0.01 vs control, Figure 4). There was a non-significant attenuation in the increase in LVEDV (64.8 ml (60.7, 71.3) to 83.5ml (74.7, 90.8), p=0.14 vs control, Figure 5). Unlike the control group, the ejection fraction remained stable (30.5% (28.4, 33.4) to 32.9% (28.6, 36.9), p=0.04 vs. control,
Figure 6). As with control animals, the infarct size fell over time in the cell-treated group (10.9% (9.8, 12.5) to 7.3% (6.5, 8.8), p=0.002 p=NS compared to control group). Infarct volume fell from 5.4 ml (4.4, 7.1) at 6 weeks to 4.4 ml (2.9, 5.0) at 30 weeks (p=0.004, p=NS compared to control group).

A secondary aim of the study was to examine whether injection of mCSCs in the body of the infarct and vCSCs at the site of coronary ligation would lead to an enhanced recovery of function compared to mCSCs at both sites. Examination of MRI results did not reveal any between group differences comparing animals treated with mCSCs at both sites (n=6) and those treated with vCSCs (n=7) at the upper injection site and mCSCs at the lower site.

Pathology: All hearts had overt well-healed myocardial infarcts with areas of scarring showing variable degrees of dense fibrosis, focally admixed with adipose tissue and residual adjacent hypertrophied myocytes, and with scattered primarily mononuclear cell inflammation; epicardial injection sites were identifiable by the presence of suture and foreign body granuloma formation. Vascularity ranged from sparse, to focal capillary and arteriolar proliferation, to larger well-developed post-inflammatory vessels. There was no relationship identified between the nature of the injections (control vs. cell treated preparations or between CSC groups) and the characteristics, density, or distribution of fibrosis, adipose tissue, or vessels.

In three animals, distinct epicardial-based collections of cells were identified. These collections of cells were identified in two animals that received control PBS injections only, and in one animal receiving mCSCs and vCSCs. In all instances the cells appeared to originate adjacent to sutures used to seal the injection sites.
One lesion in a control animal measured 0.5 cm in greatest dimension, and was not grossly apparent, merging imperceptibly with adjacent fibrosis; the other case in a control animal presented as a firm, rubbery, expansile epicardial-based lesion measuring 4.5 x 4 x 3.5 cm (supplemental data). The large infiltrating cells did not stain with reagents specific for α-actinin, sarcomeric actin, desmin, or HHF35, although entrapped cardiomyocytes and smooth muscle cells within vessels could be identified. These cells also did not show immunohistochemical staining for CD34, cytokeratin, vWF, CD19, CD20, CD45, CD163, c-Kit, S100, or myf-4. After exhaustive work-up, the lesions can best be described as atypical histiocytoid proliferations of uncertain biologic potential. Since these atypical collections of cells occur at the suture sites and after PBS injection only, it is most likely that these lesions represent an unusual reactive lesion resulting from the experimental manipulations.

In addition, one of the experimental post-infarct animals receiving mCSCs and vCSCs also exhibited a sub-population of infiltrating cells that by immunofluorescence and immunohistochemistry expressed markers consistent with immature cardiomyocytes, including cardiac α-actinin, sarcomeric actin, desmin, and HHF35; cells within these foci also occasionally expressed Nkx2.5, and very rarely connexin 43 and c-Kit (CD117). These cells did not show immunohistochemical staining for CD34, cytokeratin, vWF, CD19, CD20, CD45, or CD163. Notably, these cells were not identified in the other CSC-injected hearts despite extensive sampling.
Discussion

Canine models of ischemia, myocardial infarction, and heart failure have been previously reported. However, the presence of vigorous collateral circulation in the canine has been considered to limit their use as models for progressive adverse ventricular remodeling. Others have approached this limitation by causing repetitive global infarcts with injection of microspheres\textsuperscript{23}. We report the development of a canine model of chronic myocardial infarction with continued adverse remodeling in the late phase (6 to 30 weeks). At 6 weeks, animals showed depressed EF in the range of 30-35\% (normal range 50 to 70\%\textsuperscript{19,20}) increased systolic and diastolic volumes. Other groups have reported large infarct creation in the canine with ligation of the proximal LAD, diagonals, and collaterals from other territories but have limited the duration of follow-up to 12 weeks\textsuperscript{24}. In our study of large infarcts, we confirm adverse LV remodeling at 6 weeks and 30 weeks demonstrating continued increase in LV volumes and a further non-significant decrease in EF. The choice of the canine model in this study was made due to the well-characterized nature of CSCs in this species. It is important to note, however, that other species of large animals have been utilized as models of ischemic heart failure including pigs and sheep\textsuperscript{25}. Pig myocardial infarction models have been used and have the advantage of a lesser developed collateral circulation compared to dogs. Similarly, selective ligation of coronary arteries in the sheep has been used to study heart failure and has found particular value in the creation of models of ischemic mitral regurgitation. The control group in our study fulfilled our objective by demonstrating progressive adverse left ventricular remodeling.

We now report that in this canine model of chronic infarction and late adverse ventricular remodeling, the direct injection of myocardial CSCs leads to a durable improvement in ventricular size and EF. Most prior animal studies have focused on the use of stem cells in the
acute or subacute phase of myocardial infarction. However, the cardiovascular milieu is markedly different in the chronic infarct in terms of inflammation, cytokines, growth factors, and adhesion molecules possibly having an effect on the success of stem cells to engraft and flourish. Tang et al. investigated the effect of CSC injection in the chronically infarcted rat heart utilizing a model of cell injection 30 days post infarct. A single clonogenic c-kit\textsuperscript{pos} cell expressing enhanced green fluorescent protein (EGFP) was used to develop a c-kit\textsuperscript{pos}-EGFP\textsuperscript{pos} clone, which was used in these studies. At 35 days post injection, the authors found improvement in LV function, with an increase in viable myocardium and less fibrosis associated with injection of CSCs. Prior studies of CSC injection in the canine model have focused on injection following acute myocardial infarction and have utilized cross-species cells in immune-suppressed animals with relatively short follow-up. We studied the effect of autologous stem cell injection in the chronically infarcted canine heart (6 weeks after coronary ligation) at a time point when adverse remodeling was already apparent and found that the injection of ~1.6 million CSCs had a favorable effect on the late phase of cardiac remodeling in the chronically infarcted canine heart.

A common strategy employed in animal models of cell therapy for myocardial regeneration is the use of cross-species cells, typically human cells in an animal recipient. This strategy allows for the tracking of cells through immuno-cytochemical methods to determine cell fate. However, this practice requires the use of immunosuppression to limit xenogenic cell rejection. In turn, this limits the duration of experiments and thus little is known about the durability of beneficial effects of cardiac regenerative therapies. Use of autologous cells circumvents this problem and has been the practice in human studies of stem cells in cardiac repair. We utilized autologous CSCs in this study to extend the period of observation to 30
weeks and found a significant improvement in LV volumes and LVEF compared to control at this time point suggesting a durable result from CSC injection.

There are several human trials that have investigated the use of intra-coronary autologous bone marrow mononuclear cells (BMCs) in the setting of acute myocardial infarction (within 1 week) showing improvement in LV ejection fraction. However, the recently published Late TIME trial investigated the use of BMCs at 2 to 3 weeks post infarction and found no beneficial effects on global or regional function. Other investigations have focused on cardiac-derived progenitor cells. The recently published CADUCEUS Phase 1 trial utilized cardiosphere-derived cells cultured from endomyocardial biopsy specimens from patients with ischemic cardiomyopathy after recent MI. These cells, notable for the presence of the cell-surface marker CD105, were given via intracoronary injection to the region of infarct. At 6 months there was a reduction in scar mass as assessed by MRI although no change in LVEF or ventricular volumes. The SCIPIO trial (a Phase 1 study) investigated the use of c-kit+ CSCs in the treatment of patients with ischemic cardiomyopathy 4 months post cardiac surgical bypass. In this group of patients with chronic ischemic dysfunction, intracoronary mCSCs improved LVEF from 30.3±1.9% before mCSC infusion to 38.5±2.8% 4 months after infusion. In addition to the species and other obvious differences between the SCIPIO trial and our animal study, our model was the dynamic phase of adverse remodeling following experimental infarction as opposed to the stable reduced EFs in patients undergoing elective revascularization enrolled in the clinical trial. Although we did not observe a statistically significant improvement in EF in our animal study, we consider the attenuation of adverse remodeling using autologous CSCs in our coronary ligation model to be supportive of beneficial cardiac effects of these cells.
The durability of effect of stem cell cardiac therapy in humans is also an important issue. For example, in the BOne MarrOw Transfer to Enhance ST-Elevation Infarct Regeneration (BOOST) trial, patients underwent intracoronary infusion of unselected, autologous, BMCs 4.8±1.3 days after having undergone successful percutaneous coronary intervention for acute MI. Using cardiac magnetic resonance imaging to evaluate ventricular function, the authors reported a statistically significant improvement in ventricular function in the patients assigned to BMC infusion at 6±1 month after random treatment. However, at the 18-month evaluation, the difference between groups was no longer significant. These findings raise the important issue of durability of results from single administration of stem cells emphasizing the need for long term studies.

A secondary aim of the current study was to examine the potential effect of preferentially delivering vCSCs at the proximal site of injection. Bearzi et al. identified a human subset of CSCs committed to a vascular lineage and identified by the presence of the cell marker KDR (vCSCs). These human cells (50,000), when injected into canine myocardium at the site of an experimentally induced critical stenosis, were found to produce small, medium, and large coronary arteries and led to functional improvement of ischemic myocardium. In our study, there was no signal of a difference in MRI measures of cardiac function, volumes, or in tissue examination by a cardiac pathologist. Given the small number of overall subjects in the current study (6 with mCSCs, 7 with vCSCs at upper zone), we cannot exclude a possible salubrious effect of these cells that was not statistically identifiable in this small cohort. In addition, the canine myocardium has a well-established capacity for development of vigorous collaterals which may make this model not as predictive of the potential of a specific vascular progenitor cell in ischemic regions in humans.
The atypical histiocytoid proliferations of uncertain biologic potential identified at the site of injections in 2 animals receiving control PBS injections and in 1 animal who received mCSCs and vCSCs were an unexpected finding. These unusual reactions are interpreted as likely sequelae of experimental manipulation and raise the concern for potential unintended biologic consequences of direct myocardial injection. We chose to utilize a method of direct injection of CSCs into the myocardium through an epicardial approach. Other investigators have utilized an endovascular approach with specialized catheters for injection and usually in conjunction with electro-mechanical mapping. Even more intriguing is the recent evidence that a direct coronary injection route leads to cell engraftment in animals\textsuperscript{31} and functional improvement in both animals\textsuperscript{31} and humans\textsuperscript{13}. Such an approach has obvious appeal due to its less invasive approach and specifically in our study may have led to less inflammatory reactions.

Limitations: The chief limitation of the current study is the lack of ability to track cells and to therefore comment on mechanisms of improvement in remodeling. Prior studies of CSCs in animal models of infarct have demonstrated improvement in cardiac function attributable to the differentiation of delivered cells into functioning myocytes\textsuperscript{8}. Some studies also suggest beneficial paracrine effects including the capacity of delivered cells to stimulate endogenous CSCs\textsuperscript{32}. The use of autologous cells allowed for the long duration of follow-up in these animals and was the principal aim of this study. The use of cross-species cells would have allowed for tracking of cells by immunohistochemistry but the immune-suppression necessary for such a strategy was not felt to be practical for such a long period. The current study was designed to pragmatically address whether CSC therapy could alter the late phase of adverse ventricular remodeling following myocardial infarction and was not designed to allow determination of specific mechanistic insights. Therefore, it is impossible to ascribe the favorable effects on LV
remodeling to engrafted cells or combined to paracrine effects. This study was not designed to compare different modalities or techniques within the field of regenerative therapy but rather to study the effect of CSCs in this model. Other limitations of the study include lack of baseline (prior to infarction) and interim time points for study. These time points were not studied in order to limit the anesthetic exposures of the animals.

**Conclusions**

We report a large animal model of late (6 to 30 week) LV remodeling following myocardial infarction with increases in LVESV and LVEDV and a reduction in EF. In this study, we demonstrated that intramyocardial injection of autologous CSCs favorably altered the biology of this process and attenuated long term adverse cardiac remodeling and preserved LV function.

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Table. Cardiac parameters for control and cell-treated groups at 6 and 30 weeks post infarction.

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<th>Control (n=6)</th>
<th>Treated (n=13)</th>
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<td>6 weeks</td>
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<tr>
<td>Weight (kg)</td>
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<td>LVESV (ml)</td>
<td>51.3 (43.3, 57.4)</td>
<td>76.1 (72.0, 82.4)</td>
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<td>LVEDV (ml)</td>
<td>78.5 (69.7, 86.1)</td>
<td>99.2 (97.1, 100.4)</td>
<td>83.5 (74.7, 90.8)</td>
</tr>
<tr>
<td></td>
<td>p=0.03</td>
<td>p=0.03</td>
<td>p=0.002</td>
</tr>
<tr>
<td>Infarct size (%)</td>
<td>11.7 (8.6, 17.4)</td>
<td>9.7 (5.0, 11.2)</td>
<td>10.9 (8.8, 12.5)</td>
</tr>
<tr>
<td></td>
<td>p=0.03</td>
<td>p=0.03</td>
<td>p=0.002</td>
</tr>
<tr>
<td>Infarct Volume</td>
<td>6.2 (4.2, 7.6)</td>
<td>4.8 (4.6, 7.7)</td>
<td>5.4 (4.4, 7.1)</td>
</tr>
<tr>
<td></td>
<td>-0.7 (-1.4, 0.2)</td>
<td>p=0.17</td>
<td>4.4 (2.9, 5.0)</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>35.2 (27.9, 38.7)</td>
<td>26.4 (22.0, 31.0)</td>
<td>30.5 (28.4, 33.4)</td>
</tr>
<tr>
<td></td>
<td>-9.4 (-18.2, 0.4)</td>
<td>p=0.12</td>
<td>32.9 (28.6, 36.9)</td>
</tr>
<tr>
<td></td>
<td>p=0.04</td>
<td>p=0.004</td>
<td>p=0.31</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>21.0 (20.0, 23.0)</td>
<td>22.0 (18.0, 22.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p=0.70</td>
<td></td>
<td>0.70</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Experimental design from infarct creation through terminal hemodynamics and pathologic examination with numbers of animals remaining in study at each time point.

Figure 2. Picture representing infarct model. Red x’s mark sites of coronary ligation of LAD and branches. Shaded zone represents infarct area. The arrows show the path of needle insertion and administration of cells radiating from the central needle insertion sites.

Figure 3. Representative photograph of a serially transversely sectioned heart revealing antero-apical infarction.

Figure 4. LVESV over time in both control and cell-treated animals. Wilcoxon signrank tests were performed to analyze the data within each group and p-values are provided on chart. Using the Wilcoxon rank sum test, the control group was compared to the cell treated group showing a p-value of 0.01. Shaded zone shows normal values21, 22. Boxplots show the median and interquartile range for each timepoint.

Figure 5. LVEDV over time in both control and cell-treated animals. Wilcoxon signed rank tests were performed to analyze the data within each group and p-values are provided on chart. Using the Wilcoxon rank sum test, the control group was compared to the cell treated group showing a p-value of 0.14. Shaded zone shows normal values21, 22. Boxplots show the median and interquartile range for each timepoint.

Figure 6. LVEF over time in both control and cell-treated animals. Wilcoxon signed rank tests were performed to analyze the data within each group and p-values are provided on chart. Using the Wilcoxon rank sum test, the control group was compared to the cell treated group showing a p-value of 0.04. Shaded zone shows normal values19, 20. Boxplots show the median and interquartile range for each timepoint.
Figure 5

LVEDV (ml)

Control

Treated

6 week
30 week

P = 0.14 control vs treated

P = 0.03

P = 0.002
Figure 6

Control vs Treated

LVEF (%)

6 week 30 week

P = 0.04 control vs treated

P = 0.12

P = 0.31
Effect of Cardiac Stem Cells on Left Ventricular Remodeling in a Canine Model of Chronic Myocardial Infarction


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Supplemental Materials

Primary antibodies were reacted at the indicated dilutions at room temperature for 40-60 minutes or overnight at 4°C; secondary reagents were DAKO Envision mouse or rabbit antibodies (Carpinteria, CA) incubated at 1:100 dilution for 30 minutes at room temperature.

Immunohistochemical staining for CD34 (anti-human epitope), HHF35 (muscle-specific actin), Ki-67, c-Kit (CD117), CD45, or S100 was performed without antigen retrieval; immunohistochemical staining for desmin, CD163, CD34 (anti-canine epitope), von Willebrand factor (vWF), cytokeratin, or myogenin (myf-4 transcription factor) was performed after antigen retrieval using citrate buffer pressure cooking. Anti-desmin antibody (Sigma, St. Louis, MO) was incubated at 1:5000 dilution; anti-HHF antibody (DAKO) was incubated at 1:400; anti-CD34 antibody (DAKO) or anti-canine CD34 antibody (BioLegend, San Diego, CA) was incubated at 1:400 or 1:50, respectively; anti-cytokeratin antibody (MyBioSource, San Diego, CA) was incubated at 1:300 dilution; anti-canine CD45 antibody (AbDSerotec, Raleigh, NC or Leukocyte Antigen Biology Laboratory, Davis, CA) was incubated at 1:10-1:1000 dilutions; anti-canine vWF antibody (LifeSpan BioSciences, Seattle, WA) was incubated at 1:4000 dilution, anti-CD163 antibody (Novocastra, Leica Microsystems, Inc. Buffalo Grove, IL) was incubated at 1:250; anti-CD117 antibody (c-Kit; DAKO) was incubated at 1:250; anti-S100 antibody (DAKO) was incubated at 1:1000; anti-myogenin antibody was incubated at 1:50; anti-Ki-67 antibody (DAKO) was incubated at 1:250.
Figure 1. Representative histology (40X) of scars at the sites of injection in animals receiving myocardial stem cells (Panel A), or phosphate-buffered saline only (Panel B). Regardless of the nature of injection, there was variably dense fibrosis, focally admixed with adipose tissue and residual adjacent hypertrophied myocytes, with scattered primarily mononuclear cell inflammation; epicardial injection sites (*) were identifiable by the presence of suture and foreign body granuloma formation.
Figure 2. Gross (A) and microscopic (B) images from an animal that received only a saline injection. There is a 4.5 cm infiltrative nodule of large irregular cells with irregularly clumped chromatin and an associated mixed inflammatory cell infiltrate. The infiltrating cells did not stain with reagents specific for α-actinin, sarcomeric actin, desmin, or HHF35, although entrapped cardiomyocytes and smooth muscle cells within vessels could be identified. These cells also did not show immunohistochemical staining for CD34, cytokeratin, vWF, CD19, CD20, CD45, CD163, c-Kit, S100, or myf-4. B, 400X.
Figure 3. FACS analysis of canine c-kit positive cardiac stem cells. SSC, side scatter.