Massive mechanical loss of microspheres with direct intramyocardial injection in the beating heart: Implications for cellular cardiomyoplasty

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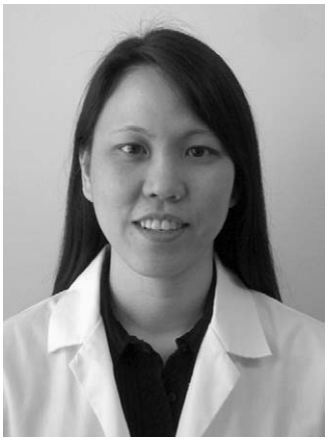

Dr Teng

Objective: Direct intramyocardial injection is a common route of donor cell administration for myocardial cell therapy. Studies have demonstrated a significant and rapid loss of implanted cells, which is thought to be biologically caused. We hypothesized that mechanical loss of cells from the contracting myocardium might actually be the main culprit.

Methods: Intramyocardial injections of fluorescent microspheres (10 μm) were carried out in both small and large animal models. The hearts of Lewis rats (250-350 g) received 3×10^6 microspheres injected into the left ventricular myocardium. Rats were divided evenly between two experienced operators. The nonbeating ($n = 2$) and beating ($n = 5$) hearts of piglets (7.5-7.8 kg) received 3×10^6 microspheres. The hearts were excised within 10 minutes, and the microspheres retained in the myocardium were quantified with fluorescent flow cytometry.

Results: In the beating-heart rat model, the microsphere retention rates after a single injection were similar with and without purse-string occlusion of needle puncture sites and slightly lower than after multiple site injections (6.19% $\pm$ 4.05% vs 5.44% $\pm$ 5.66% vs 8.83% $\pm$ 3.29%). There were no significant operator-dependent differences. The retention rates in beating porcine hearts were higher than those in the rats ($P < .05$) but markedly lower than those in nonbeating porcine hearts (11.1% vs 67.4%).

Conclusion: Mechanical leakage and washout may account for a major portion of cell loss after cell implantation, and efforts aimed at reducing mechanical loss in the beating heart may yield a greater benefit than those targeting biologic loss alone.

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Survival of cells after intramyocardial injection is crucial to the efficacy of therapeutic cell transplantation. In an attempt to increase the number of cells surviving after injection, many researchers have targeted cell deaths (due to apoptosis, ischemia, free radical formation, etc). However, recent studies suggest a massive loss of cells in the first minutes after injection.[1-4] In light of the early time frame in which this loss takes place, it is unlikely to be accounted for solely by cell death.

Cells can be delivered in a variety of ways, and one of the most common methods is direct intramyocardial injection. The technique of injection may result in mechanical loss, in the form of cells leaking from the site through the puncture hole, cells retained in the syringe, or vascular washout. Because the heart differs from other organs in that it is constantly contracting, it is possible that this may contribute to the mechanical loss by squeezing the injected cells out of the myocardium. As a result, the cells retained in the myocardium immediately postinjection represent only a fraction of those initially implanted. It is from this subset that biologic loss and gain, through cell death and proliferation, can then affect the quantity of surviving cells.

Small animal models have been used extensively to examine the effects of cellular implantation in the myocardium. Because of the small size of the heart, relative thinness of the ventricular wall, and rapid cardiac contractions, mechanical loss may be more pronounced in smaller animals than in larger animals. Thus examining mechanical loss in the small animal model is relevant to much of the basic science studies being done, whereas the larger animal model may be more clinically relevant.

Many studies have attempted to limit the biologic loss from cell death by focusing on measures such as antiapoptotic[5,6] or angiogenic gene therapies,[7] as well as heat shock and anti–free radical treatments.[8] Although limiting cell death may be important, if the initial decrease in cells from mechanical loss leaves only a fraction of the cells within the myocardium postinjection, then keeping this small fraction alive may not be as effective as simply retaining more cells by limiting mechanical loss. Many studied have focused on the biologic component of cell loss; however, few have addressed the contribution of mechanical loss in determining the final number of surviving cells postimplantation. Thus the objectives of this study were as follows: (1) to quantify mechanical loss during direct intramyocardial injection, (2) to compare small and large animal models, (3) to quantify the contribution of myocardial contraction to mechanical loss, and (4) to define a model for quantifying mechanical cell loss that may be used as a tool to test and develop better techniques of cell implantation for myocardial regenerative therapy.

Methods

All experiments were performed in accordance with the guidelines set forth by the Canadian Council on Animal Care.

Each aliquot of microspheres (from Interactive Medical Technologies, Ltd (Irvine, Calif) contained 3 million microspheres (±3.75%) measuring 10 μm in diameter and suspended in 150 μL or 800 μL (for rat or pig injections respectively) saline solution with 0.05% polysorbate 80 and 0.01% thimerosal.

Rat Heart Intramyocardial Injections

Male Lewis rats weighing 250 to 350 g (n = 36) were placed under general anesthesia with 5% isoflurane in an induction chamber. The rats were then intubated with an 18-gauge catheter and mechanically ventilated. Anesthesia was maintained with 3% isoflurane. A left thoracotomy was performed to provide access to the heart. The pericardium was entered, and an injection of 100 μL microsphere solution was prepared with a 0.5-mL tuberculin syringe and a 28-gauge needle. In the first group of rats (n = 12), a single injection directly into the myocardium was used to administer the microspheres. For the second group (n = 12), a U-stitch of 6-0 Prolene (Ethicon, Inc, Somerville, NJ) was made in the epicardium before injection. The injection was made with the needle passing through the center of the U-stitch, and the epicardial opening of the needle tract was closed by tying down the stitch as the needle was withdrawn. The third group of rats (n = 12) received 100 μL of solution divided into 3 intramyocardial injections given in separate areas on the anterolateral wall of the heart with the same syringe. After each injection, the surgeons would record the subjective assessment of how successful the injection was on a scale of 0 to 10 (0 being an injection where the surgeon felt that all of the microsphere solution was injected too deeply or superficially, and 10 being an injection where the surgeon felt that all of the microsphere solution was injected perfectly into the myocardium). The number of rats in each group was divided evenly between the two surgeons.

Rat hearts were removed within 10 minutes after injection. Hearts were flushed with phosphate-buffered saline solution through the aorta in a retrograde fashion to remove excess microspheres that might have been retained in the ventricular chamber rather than in the myocardium. The surface of each heart was also washed in phosphate-buffered solution. The whole heart was then individually placed in a 15-mL tube and sent to Interactive Medical Technologies Laboratory for processing. In addition, each syringe used for injection was flushed with saline solution, and the saline flush was collected in the respective vials from which each aliquot was drawn. Each of these vials was also sent back to Interactive Medical Technologies Laboratory to count the number of microspheres that had not been injected. Given that 3 million microspheres were originally present in each vial, subtracting the sum of the microspheres left in a vial plus the number of microspheres left in the syringe from the initial 3 million would give the actual number of microspheres injected. Both the tissue samples and vials of unused microspheres were processed for immediate shipment to Interactive Medical Technologies, Investigative Partner Services, where individual tissue samples were digested in alkaline solution. Microspheres were then collected on a filter, resuspended, and quantified with fluorescent flow cytometry.

Porcine Heart Intramyocardial Injections

Landracer × Yorkshire × Duroc pigs weighing 7.5 to 7.8 kg were placed under general anesthesia, intubated, and mechanically ventilated. A sternotomy was performed to provide access to the heart. For the beating-heart group (n = 5), the pericardium was entered and an injection of 700 μL of microsphere solution was prepared with a 1.0-mL tuberculin syringe and a 28-gauge needle. A single dose of orange microspheres was injected directly into the myocardium of the left ventricle. For the multiple injection samples, 700 μL of violet microsphere solution was divided into three intramyocardial injections given in separate areas on the left ventricle with the same syringe. In the nonbeating-heart group (n = 2), the heart was extracted from the chest cavity of the pig, and a single injection of microspheres was carried out directly into the arrested myocardium of the left ventricle.

The porcine hearts were removed within 10 minutes after injection. The left ventricle was excised and placed in a 50-mL tube and sent to Interactive Medical Technologies Laboratory for processing. The quantity of microspheres in the tissue was determined and calculated as described previously. The orange and violet microspheres were counted separately.

Statistical Analysis

All values were expressed as mean ± standard error of mean. Analysis of variance (F-test) was used to calculate the statistical

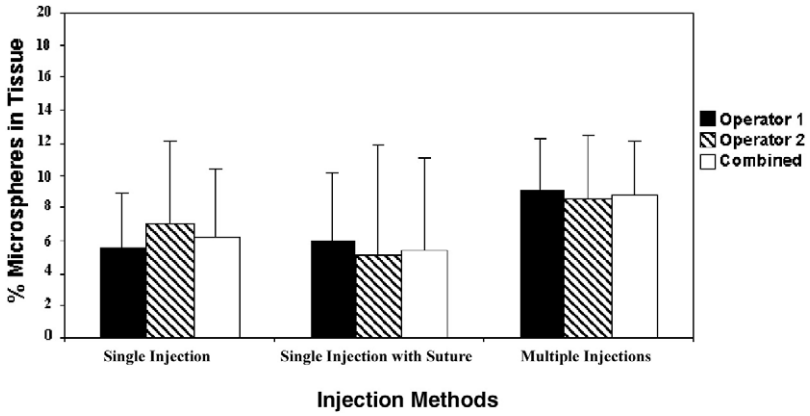

Figure 1. Microsphere retention rates in beating-heart rat model. *Combined* indicates that data from operators 1 and 2 are pooled together.

significance of differences between the means of groups. To combine the data obtained from the two operators, the tests for normality and for equal variance were performed. When analysis of variance was significant, post hoc analysis with the Bonferroni correction and selected *t* tests were to be performed.

Results

Rat Heart Intramyocardial Injections

There was no difference in the rate of microsphere retention between the two operators who performed the injections (Figure 1). Also of note, there was poor correlation between the operators' subjective ratings of the quality of injection at the time of implantation and the rates of microsphere retention (Figure 2).

Because the tests for the normality and for equal variance for the data obtained from the two operators were positive, they were combined for further analysis. The combined microsphere retention rates were similar in the single-injection group and the suture ligation group (6.19% ± 4.05% vs 5.44% ± 5.66%, respectively). The multiple-injection group had a slightly higher retention rate (8.83% ± 3.29%; Figure 1), although these differences did not reach statistical significance according to analysis of variance.

Porcine Heart Intramyocardial Injections

The percentage of microspheres retained in the porcine heart post injection was significantly higher than in the rat heart ($P < .05$; Figure 3). The microsphere retention rates in the porcine hearts also showed a trend towards a higher retention rate with the multiple-injection method; however, such a difference did not reach statistical significance.

In the beating heart, the percentage of microspheres retained was markedly less than in the nonbeating heart (Figure 4). In the porcine hearts, the microsphere retention rate in the noncontracting heart was almost 7 times that in the beating heart.

Discussion

Recently, numerous basic and clinical studies have indicated that myocardial implantation of various stem cells and progenitor cells may improve cardiac function in hearts with myocardial damage. Among many unanswered issues in cellular cardiomyoplasty today is the question of how to determine the optimal quantity of cells to be implanted to obtain the maximal clinical benefits. To elucidate such a dose-response relationship in myocardial cell therapy, the questions of implantation technique and the subsequent intramyocardial cell retention and survival rates need to be examined. In many reported studies, the number of surviving cells appeared to be quite small, such that often in the histologic sections surprisingly few labeled implanted cells were found in various experimental models.

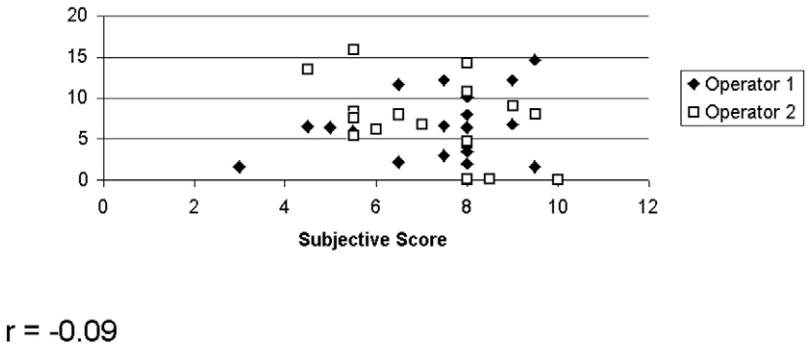

Figure 2. Beating-heart rat model, testing possible role of implant skills on microsphere retention rate.

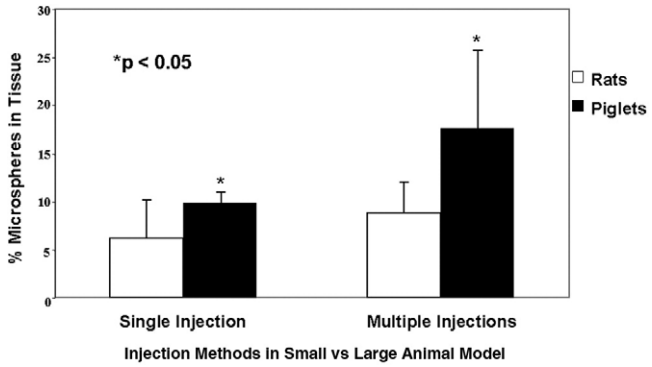

Figure 3. Microsphere retention rates in small animal (rat) versus large animal (pig) beating-heart models.

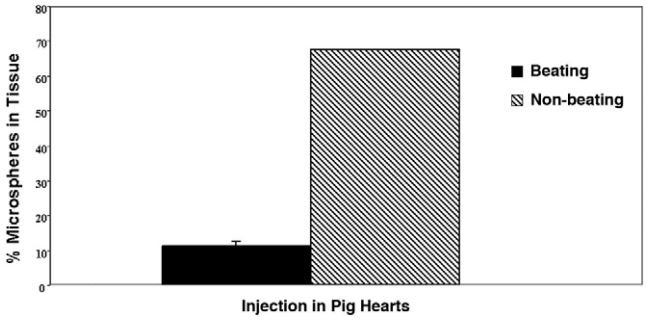

Figure 4. Microsphere retention rates in beating versus nonbeating porcine hearts.

One of the most common techniques of cell implantation into the myocardium in both experimental and clinical studies is the direct injection of the cells through the epicardium, or through the endocardium in large animal models and cardiac patients with a transvascular catheter approach. In a few quantitative studies in small animal models, very low numbers of cells appeared to have survived days and weeks after the implantation.[1] The prevailing assumption for the cause of such a low cellular survival rate is the death of these cells after implantation. Because these cells are often implanted into an ischemic myocardium, inadequate myocardial blood perfusion has been thought to be a major culprit. Other important mechanisms of biologic cell death suggested include the damage caused by free radicals and apoptosis. Thus numerous studies have been carried out to counter such pathogenic processes to minimize cell death and salvage a larger portion of implanted cells.[2-8] Implicit in these studies was the assumption that virtually all the cells injected into the myocardium were retained in situ and were then gradually depleted by various biologic processes.

There is, however, increasing evidence that such an assumption may not be valid. In a number of studies in which the quantity of implanted cells within the myocardium was studied sequentially, it was shown that a large portion of the cell loss occurred shortly after the cells were injected directly into the myocardium. Suzuki and colleagues[4] found that only 44.8% of skeletal muscle precursor cells survived 10 minutes after implantation, a figure that had steadily decreased to 14.6% by 24 hours and to 7.9% by 72 hours. Proliferation of the surviving cells began after 24 hours, increasing cell numbers to 15.5% at 24 hours and 24.4% at 72 hours. Thus there seems to be three phases of changes in cell numbers after implantation: phase I, a rapid and massive loss of cells immediately after cell implantation, followed by phase II, a period of gradual decay, and finally in some cases phase III, with some increase in cell numbers. We postulate that these three phases are caused by different mechanisms. The phase I rapid decrease in the quantity of implanted cells is likely to be mechanical, a result of cell leakage through the injection tract or a rapid washout through the coronary venous system, which was disrupted by the needle puncture allowing entry into the general circulation. This scenario is consistent with the findings of many investigators[9,10] that the implanted labeled cells are often found in large quantities in various organs, such as the lung and the liver. The phase II gradual decay of implanted cells is likely to be biologic in nature, as has been demonstrated in the studies quoted here. The phase III increase in cell number is likely to be due to the divisions of implanted stem cells or progenitor cells.

The hypothesis that the phase I and phase II cell losses are based on different mechanisms is consistent with the observations reported by Muller-Ehmsen and coworkers.[1] They found that early after injection the number of retained cells correlated linearly with the number of cells injected, but at the phase II period there was no correlation between the number of injected cells and the number of surviving cells. Different kinetics of cell loss strongly suggests different underlying mechanisms.

The magnitude of microsphere retention rate reported here is lower than those reported by some other studies that used cell markers (eg, Suzuki and colleagues,[4] 44.8%, and Muller-Ehmsen and coworkers,[1] 57%). This may be due to different experimental models and quantitating techniques used. It is possible that cells injected into noncontractile scar tissue may be retained better than those injected into contractile border zone of myocardial infarction. The technique of quantitating the fluorescent microspheres in the tissue used in our study has been validated in numerous reports in which this technique has been used to study myocardial tissue blood perfusion. Certainly the microspheres, unlike the cells, do not have surface adhesion molecules or plasticity in shape, which may play a role in retention rate. Nevertheless, the rationale for using microspheres that correspond closely in size to the implanted cells is to remove

completely the possibility of cell death from biologic factors, thus isolating the mechanical mechanism of cell leakage and washout.

Our rat study was carried out because this small animal model has been used extensively in basic research on myocardial cell therapy. The very low retention rate of microspheres in the rat hearts relative to that seen in the porcine model (Figure 3) may be related both to the technical challenge and to physiologic characteristics of dealing with a very small rat heart with thin myocardium that is contracting rapidly during and after implantation. Our finding that the purse-string suture on the epicardium around the puncture hole, which was tied immediately on removal of the needle, made no significant difference to the retention rate suggests that the backward leakage played a relatively minor role. This in turn suggests that washout of the implanted microspheres, perhaps assisted by the myocardial contractile force, played a major role in their loss from the injection site. The injections of microspheres were carried out by two cardiac surgery residents with training in surgical techniques, both of whom had experience for nearly a year in the research laboratory in injecting cells into rat myocardium (Figure 1). There was no difference between data obtained by these two operators. That their subjective judgment regarding the technical quality of cell implant in each animal correlated poorly with the quantity of microspheres lost is also of interest, suggesting that this massive loss may not have been due primarily to technical difficulties (Figure 2).

The large animal study was carried out because the size of the porcine heart, the myocardial thickness, and the cardiac contraction rate are closer to those of the human heart. Thus the data obtained may be more relevant to human clinical implantations. The importance of myocardial contractile force squeezing these microspheres to leak back or to be washed out was fully illustrated when these microspheres were injected into noncontractile porcine myocardium (Figure 4). Although the sample size was small, the observation that myocardial microsphere retention in a noncontracting myocardium was 7 times that for a similar heart in active contraction strongly supports this notion.

Our finding that multiple small bolus injections, and particularly injection into a noncontracting myocardium, improved the retention rate of cells could be clinically relevant. For example, a patient undergoing cardiopulmonary bypass and cardioplegic arrest would have a motionless myocardium. Thus when cell therapy is to be combined with a surgical procedure such as on-pump coronary bypass surgery, it could be advantageous to implant the cells during cardioplegia, rather than after removal of aortic crossclamp. To confirm this possible strategic advantage, however, further studies are required to ascertain that the microspheres are not rapidly washed out on recovery of cardiac contractions. Thus this experimental model of using microspheres to study the mechanical leakage could be a simple and useful tool to further improve cell transplantation techniques, optimizing application in clinical patients.

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References

1. Muller-Ehmsen J, Whittaker P, Kloner RA, Dow JS, Sakoda T, Long TI, et al. Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium. *J Mol Cell Cardiol.* 2002; 34:107-16.
2. Retuerto MA, Schalch P, Patejunas G, Carbray J, Liu N, Esse K, et al. Angiogenic pretreatment improves the efficacy of cellular cardiomyoplasty performed with fetal cardiomyocyte implantation. *J Thorac Cardiovasc Surg.* 2004;127:1041-9.
3. Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. *J Mol Cell Cardiol.* 2001;33:907-21.
4. Suzuki K, Murtuza B, Beauchamp JR, Brand NJ, Barton PJ, Varelaa-Carver A, et al. Role of interleukin-1β in acute inflammation and graft death after cell transplantation to the heart. *Circulation.* 2004; 110(11 Suppl 1):II219-24.
5. Fujii T, Nagaya N, Iwase T, Murakami S, Miyahara Y, Nishigami K, et al. Adrenomedullin enhances therapeutic potency of bone marrow transplantation for myocardial infarction in rats. *Am J Physiol Heart Circ Physiol.* 2005;288:H1444-50.
6. Liu TB, Fedak PW, Weisel RD, Yasuda T, Kiani G, Mickle DA, et al. Enhanced IGF-1 expression improves smooth muscle cell engraftment after cell transplantation. *Am J Physiol Heart Circ Physiol.* 2004;287: H2840-9.
7. Yau TM, Li G, Weisel RD, Reheman A, Jia ZQ, Mickle DA, et al. Vascular endothelial growth factor transgene expression in cell-transplanted hearts. *J Thorac Cardiovasc Surg.* 2004;127:1180-7.
8. Suzuki K, Smolenski RT, Jayakumar J, Murtuza B, Brand NJ, Yacoub MH. Heat shock treatment enhances graft cell survival in skeletal myoblast transplantation to the heart. Circulation 2000;102(19 Suppl 3): III216-III21.
9. Ishida M, Tomita S, Nakatani T, Kagawa K, Yamaguchi T, Suga M, et al. Acute effects of direct cell implantation into the heart: a pressure-volume study to analyze cardiac function. *J Heart Lung Transplant.* 2004;23:881-8.
10. Dow J, Simkhovich BZ, Keddes L, Kloner RA. Washout of transplanted cells from the heart: a potential new hurdle for cell transplantation therapy. *Cardiovasc Res.* 2005;67:301-7.