Beating and Arrested Intramyocardial Injections Are Associated with Significant Mechanical Loss: Implications for Cardiac Cell Transplantation

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Background. Cellular cardiomyoplasty is emerging as a potentially novel therapeutic option for heart failure and typically involves direct intramyocardial injection of donor cells into a beating heart. Yet, limited rates of cell engraftment remain an obstacle to be overcome before cell therapy is fully recognized. Mechanical and biological mechanisms may account for observed donor cell loss. This study examines acute mechanical loss during intramyocardial injections in beating and arrested hearts.

Materials and methods. A porcine cardiopulmonary bypass model was used. Animals underwent either beating (n = 5) or arrested (n = 5) intramyocardial injections into the left ventricle. Fluorescent microspheres were used in lieu of cells because they are biologically inert. Thirty minutes after delivery, animals were euthanized. Microspheres in cardiac and peripheral tissues were quantified using flow cytometry.

Results. Approximately 10% of microspheres were retained within the site of injection in both groups. There was no statistical difference between microsphere retention rates in either the beating or the arrested heart group. Microspheres were found in peripheral organs, pericardial fluid, and the delivery device.

Conclusions. The majority of microspheres injected intramyocardially are lost in both beating and arrested hearts. Cardiac standstill does not enhance microsphere retention. Possible mechanisms include leakage from the injection site and washout via the cardiac venous/lymphatic system. Delivery strategy will need to be modified if more cells are to be retained within the target organ.

Key Words: cardiac; cellular cardiomyoplasty; cardiopulmonary bypass; intramyocardial injection.

INTRODUCTION

Cellular cardiomyoplasty (CCM) is a novel innovative therapy that may prove to be a valuable option for patients with heart failure. CCM typically involves direct intramyocardial injection of cells to the failing myocardium in an effort to improve cardiac function, ameliorate symptoms, and ultimately prolong survival. Transplanted cells are expected to engraft, differentiate, and remodel in response to the surrounding microenvironment. The anticipated outcome is tissue regeneration and functional repair, yet in both human and animal studies, donor cell retention rates range from 1 to 15% [1–3].

To date, studies evaluating cell transplantation to treat heart failure have used direct injection or intracoronary delivery into a beating heart. However, there are data to suggest that intramyocardial injection may be the most promising technique of cell delivery [4]. Hou et al. demonstrated in an ischemic swine model the superiority of intramyocardial injection as compared to intracoronary and retrograde coronary venous delivery of indium-oxine-labeled cells [5]. Independent of the cell donor type and delivery method, cell engraftment has been very low with histological sections demonstrating few labeled implanted cells. Potential mechanisms of such low cellular survival include cell death after implantation, ischemia, and rejection. Implicit in this is the assumption that virtually all cells injected into the myocardium are retained in situ and are grad-
ually depleted by various biological processes. However, this assumption may not be valid. Recently, Teng et al. described three phases of cell loss associated with intramyocardial injections. The first phase consists of a rapid decrease in the quantity of implanted cells, resulting from leakage through the injection tract or washout through the coronary venous system, allowing entry into the general circulation [2, 6]. We therefore chose to examine this delivery method in our study.

Furthermore, to date, there has been no study comparing cell delivery and retention in beating and arrested hearts in a large animal model. In contrast to beating heart delivery, cardiac surgery provides a unique milieu for CCM. Because the myocardium is uniquely isolated during cardiopulmonary bypass (CPB), cell delivery to the myocardium may be improved relative to injection into the beating heart. It is possible that CPB with cardioplegic arrest would minimize back leak, increase myocardial dwell time, and increase retention of cells.

Thus, to better understand the mechanics of cell delivery, this study was designed to quantitatively compare the delivery of fluorescent microspheres using direct intramyocardial injection in both the beating and the arrested porcine heart with microsphere retention as the endpoint. Fluorescent microspheres were used because they do not undergo biological death and can be readily counted in all cardiac tissue. This study was designed to evaluate short-term retention, as this endpoint provides specific information regarding delivery parameters. The objectives of this study were as follows: (1) to quantify “mechanical” loss during beating heart intramyocardial injection in a clinically relevant large animal model; (2) to evaluate the effects of CPB and cardiac standstill on the efficacy of delivery and retention; and (3) to examine the peripheral (non-cardiac) distribution of microspheres. The results of this study will allow for the development of better techniques for CCM to maximize the number of delivered cells to the failing myocardium.

MATERIALS AND METHODS

Animal Care and Preparation

All experiments were performed in accordance with the Animal Care and Use Committee of the Brody School of Medicine, East Carolina University. Domestic swine (n = 10) of either sex weighing 20–25 kg were anesthetized with an intramuscular injection of Telazol (5 mg/kg)/xylazine (1 mg/kg). An intravenous catheter was placed in a right superficial ear vein to administer intravenous fluids. Arterial blood pressure was monitored by an 8-Fr catheter (Daig, Minnetonka, MN) placed into the right femoral artery. After endotracheal intubation, all animals were mechanically ventilated with continuous 2–2.5% isoflurane for the duration of the experiment. The heart was exposed through a median sternotomy and suspended in a pericardial cradle.

Fluorescent Microspheres

Fluorescent microspheres measuring approximately 15 μm in diameter were obtained from Interactive Medical Technologies (IMT, Irvine, CA). Each 2 mL aliquot contained 2 × 10^6 fluorescent microspheres suspended in thimerosal (0.01%) and Tween-80 (0.05%). Two doses of 1 mL were created from each aliquot and suspended in sterile phosphate-buffered saline.

Porcine Intramyocardial Injections

Beating Heart Group

After exposing and appropriately positioning the heart, a 1 mL insulin syringe (U-100, 29 gauge; Becton Dickinson, Franklin Lakes, NJ) was used to inject fluorescent microspheres intramyocardially into the anterior left ventricular wall (Fig. 1). Two 1 mL doses of microspheres (total of 2 × 10^6) were injected into each animal (n = 5). Animals were euthanized 30 minutes after the completion of the injections (Fig. 2).

Arrested Heart Group

After exposing the left femoral artery, an arterial cannula (14 Fr; Bard, Covington, GA) was placed. A venous cannula (28/38 Fr; Sarns, Ann Arbor, MI) was inserted into the right atrium. Animals (n = 5) were placed on normothermic CPB. Cold crystalloid cardioplegia (17 mEq KCl/L) was infused into the ascending aorta after the cross-
clamps were applied. After cardioplegic arrest, two 1 mL doses of microspheres (total of \(2 \times 10^6\)) were injected with a 1 mL insulin syringe into the wall of the anterior left ventricle. The aortic clamp was removed 5 minutes after the injections. All animals were weaned off of CPB 15 minutes after the removal of the cross-clamp. Animals were euthanized 15 minutes after separation from the CPB machine (Fig. 2).

Tissue Harvesting and Preparation

Porcine hearts were removed and divided into the following sections: (1) right ventricle and atrium; (2) anterior left ventricle; (3) posterior left ventricle; (4) left atrium; and (5) interventricular septum. Representative tissue samples were collected from the right and left lungs, liver, spleen, kidney, fluid from the pericardial well, 5 mL of blood from the inferior vena cava, and the CPB pump circuit. Furthermore, each syringe used for microsphere delivery was flushed with 5 mL phosphate-buffered saline with 0.05% Tween and this fluid was analyzed for residual microspheres. All harvested tissue was digested for flow cytometry analysis.

Tissue digestion protocol and reagents from IMT were used to process all samples. Briefly, tissue samples were weighed, cut into small pieces, and placed in a 50 mL polypropylene centrifuge tube (VWR, West Chester, PA). Process control microspheres and reagent I (E-Z Trac Ultraspheres; IMT) were added to each sample, vortexed, and placed in a dry oven at 70°C overnight. After incubation, reagent II (E-Z Trac Ultraspheres; IMT) was added to the suspension and mixed by inversion. The samples were centrifuged for 30 min at 2500 rpm; the supernatant was removed and each pellet was resuspended in microsphere counting reagent and vortexed. Samples were heated and then spun for 15 min at 2500 rpm and the supernatant was aspirated. Each pellet was resuspended in microsphere counting reagent and transferred to a 15 mL centrifuge tube using a 50 mm Pore Filter device (E-Z Trac Ultraspheres; IMT). All samples were spun for 15 min at 2500 rpm. The supernatant was removed and pellets were resuspended in 400 \(\mu\)L of microsphere counting reagent and analyzed on a flow cytometer (FACScan; Becton Dickinson) using a 488 argon laser. The percentage of microspheres retained was calculated by the formula:

\[
\% \text{ microspheres retained} = \left( \frac{\# \text{ of microspheres counted in a tissue sample}}{\# \text{ of microspheres injected}} \right) \times 100
\]

Statistical Analysis

Statistical analyses were performed using an unpaired Student’s \(t\)-test analysis to calculate \(P\) values. All values are expressed as mean ± SEM. For all tests, \(P < 0.05\) was accepted as statistically significant.

RESULTS

The intramyocardial injections were well tolerated in all animals. No ventricular arrhythmias were induced by the injections and all animals survived to euthanasia. Pigs undergoing arrested heart injections were separated from CPB without any inotropic support and remained hemodynamically stable for the remainder of the experiment (15 min). Gross postmortem examination revealed no evidence of myocardial infarction, although histological examination was not performed.

Overall, there was no statistical difference in the percentage of microspheres retained in the entire heart between the arrested and beating groups (Fig. 3). In fact, there was a trend toward greater microsphere retention within the beating heart group. Within the heart tissue, the average number of microspheres per gram tissue, counted with flow cytometry, was significantly elevated in the region of injection (anterior left ventricle) in both the beating and the arrested groups. However, microspheres were identified in all tissue samples from the heart (Table 1). A significantly greater number of microspheres per gram of tissue were found in the left atrium (63.7 ± 22.2 versus 4.3 ± 0.9) and right ventricle (96.4 ± 24 versus 21.4 ± 10.1) of the arrested hearts when compared to the tissue in the beating hearts. There was no difference in microsphere retention rate between the two groups at the site of injection (anterior left ventricle) (Fig. 4).

Microsphere washout was evaluated by examining the average number of microspheres per gram of tissue in samples taken from peripheral organs in both groups (Table 2). The average number of microspheres per gram of tissue found in the right kidney, liver, and spleen of the arrested hearts was significantly greater than similar tissue sampled in the beating hearts. There were significantly more microspheres in samples from the heart (Table 1). A significantly greater number of microspheres per gram of tissue were retained in the entire heart in the arrested heart group (\(P = 0.01\)) and right ventricle (96.4 ± 24 versus 21.4 ± 10.1) of the arrested hearts when compared to the tissue in the beating hearts. There was no difference in microsphere retention rate between the two groups at the site of injection (anterior left ventricle) (Fig. 4).

<table>
<thead>
<tr>
<th>Gram of Cardiac Tissue</th>
<th>Beating heart</th>
<th>Arrested heart</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right ventricle</td>
<td>21.4 ± 10.1</td>
<td>96.4 ± 24</td>
<td>0.01*</td>
</tr>
<tr>
<td>Anterior left ventricle</td>
<td>5562.8 ± 1059.7</td>
<td>4354.7 ± 1314.2</td>
<td>0.27</td>
</tr>
<tr>
<td>Posterior left ventricle</td>
<td>2019.5 ± 1168.9</td>
<td>199.6 ± 40.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Septum</td>
<td>44.5 ± 18.8</td>
<td>472.9 ± 199.3</td>
<td>0.05</td>
</tr>
<tr>
<td>Left atrium</td>
<td>4.3 ± 0.9</td>
<td>63.7 ± 22.2</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

* Statistically significant, \(P < 0.05\).
sphere count in the delivery device between the two groups (33,727 ± 7070 beating versus 28,031 ± 7029 arrested, P = 0.3). In evaluating the total recovery of microspheres (the entire heart plus representative tissue samples of lungs, liver, kidney, spleen, pericardial fluid, and the delivery device), the recovery yield (relative to the total injected quantity) was 16.4% in the beating group and 10.5% in the arrested group (P = 0.07).

**DISCUSSION**

Cellular cardiomyoplasty, or the transplantation of cells to the heart, has recently emerged as a potentially novel therapeutic approach to treat significant myocardial loss in an effort to improve left ventricular function and ameliorate symptoms of heart failure. Despite initial promising results, the type of donor cell, timing and approach of delivery, as well as the optimal cell dosage are among the many unanswered issues. Various donor cells have been used including adult bone marrow derived mesenchymal stem cells, skeletal myoblasts, and progenitor cells.

Using fluorescent microspheres, the focus of this study was to evaluate the mechanics of direct intramyocardial “donor cell” injections in beating and arrested hearts. Our findings indicate that there is no difference in microsphere retention rates between the beating and arrested heart. In fact, the low retention rates observed in both groups is not unexpected, as it is generally accepted that between 1 and 5% of delivered cells actually engraft into the myocardium [7]. Within the beating heart group, the low observed microsphere retention rate might relate to transport out of the myocardium via venous/lymphatic effluent, leading to entrapment within the lung, spleen, or kidney. Grossman et al. report similar findings in a porcine beating heart model [3]. They documented a retention rate of 15 ± 21% for 15-μm microspheres with direct epicardial injection. Furthermore, they observed egress of material during systolic contraction after withdrawal of the injection needle, much like we did in our study. Another study by Rezaee et al. demonstrated that 10% of delivered protein was retained within the heart in animals with ineffective shallow injections [8].

In contrast to beating heart delivery, Grossman et al. also reported on postmortem injections that demonstrated an 89 ± 60% microsphere retention rate, a significantly higher value than that of their beating heart group [3]. Furthermore, in another study, microsphere retention rate in the nonbeating heart was significantly (7×) higher than in the beating heart [2]. However, in both of these studies, injections were performed with the heart ex vivo and not in vivo during CPB with cardioplegic arrest. In contrast, our results did not demonstrate any enhancement of retention within the arrested heart. In the in situ heart, the microspheres could have moved via pulmonary artery, pulmonary venous, and systemic venous (inferior and superior vena cavae) connections still intact. Furthermore, delayed washout of the microspheres may have occurred when blood flow and cardiac contractions resumed after removal of the cross-clamp and resumption of blood flow to the heart.

In this study, we have demonstrated the substantial distribution of microspheres to visceral organs when performing localized myocardial delivery (intramyocardial injection) into a beating or arrested heart. We have also demonstrated that this phenomenon occurs shortly (within 30 min) after delivery. Pilot studies in our laboratory have shown that microsphere retention rates 5 minutes after intramyocardial injection are not significantly different when compared to retention rates 30 minutes after injection. Thus, the majority of washout and mechanical loss seem to occur within minutes after microsphere delivery. Therefore, presumably by 30 minutes all remaining microspheres should not be lost to any further mechanical cause. Future experiments with greater experimental duration will be required to validate this hypothesis. One noticeable difference between the two groups was the largely right-sided distribution of microspheres, marked by pulmonary trapping far in excess of that by the filter organs (liver, spleen) on the left-sided (systemic) circulation in the beating heart group. This suggests that microsphere delivery

**TABLE 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Beating heart</th>
<th>Arrested heart</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left lung</td>
<td>955.2 ± 290.2</td>
<td>40.3 ± 5.7</td>
<td>0.04*</td>
</tr>
<tr>
<td>Right lung</td>
<td>588.8 ± 252.9</td>
<td>42.6 ± 14.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>0.6 ± 0.58</td>
<td>23.8 ± 4.7</td>
<td>0.003*</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.2 ± 1.9</td>
<td>209.1 ± 40.8</td>
<td>0.002*</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.2 ± 2.8</td>
<td>76.2 ± 7.7</td>
<td>0.004*</td>
</tr>
</tbody>
</table>

* Statistically significant, P < 0.05.
into the beating heart is accompanied by egress of the microspheres from the myocardium primarily into the venous and/or lymphatic drainage, rather than into the arterial circulation, as opposed to the arrested group, in which immediately after injection, there is no blood circulating within the heart. Furthermore, after removal of the cross-clamp and resumption of blood flow to the heart, the lungs are not ventilated (animal on CPB). Thus, blood is shunted away from the lungs, allowing for more of the filter organs (liver, spleen) to retain the microspheres. The effects of remote organ engraftment in CCM are unknown and may be benign; however, engraftment may result in uncontrolled cellular growth, differentiation, or malignant transformation in remote organs. Thus, the potential effects of such remote delivery should be considered when designing clinical studies.

Potential limitations of this study exist. No histology was performed as all of the tissue was digested. This will be examined in future experiments. Furthermore, at least three mechanical factors can influence the retention of intramyocardially delivered cells: the rate, depth, and volume of delivery. For example, Hou et al. demonstrated that injection pressure changed delivery efficiency significantly when examining retrograde coronary venous delivery of indium-oxine-labeled cells in a porcine model [5]. Similar parameters may exist for intramyocardial injections of cells. Unfortunately, we did not examine each of these variables specifically. However, pilot studies in our laboratory indicate that volume of injectate (between 0.5 and 2.0 mL/injection) does not significantly affect the number of microspheres retained in a beating porcine heart model. Therefore, we elected to use a clinically relevant delivery device—a 1 mL insulin syringe. Another limitation is that microspheres, unlike cells, do not have surface adhesion molecules or plasticity, which may play a role in retention rates. Nevertheless, the rationale for using microspheres was to remove the possibility of “biological” death and to focus more specifically on the “mechanical” loss. Furthermore, these studies were performed in nonischemic (normal) myocardium. Direct myocardial injectate retention in ischemic or nonviable myocardium may be different than retention in normal cardiac tissue.

Future studies will examine the effects of CPB and cardioplegic arrest on direct injection and other methods of delivery using adult bone marrow derived mesenchymal stem cells labeled with quantum dot nanoparticles [9]. Perhaps, cell delivery using the intracoronary or endocardial approach might result in increased retention and engraftment. Ultimately, the optimal delivery technique will provide the most therapeutic benefit. However, the relationship between cell retention, engraftment, timing of delivery, and subsequent therapeutic benefit remains unknown. Perhaps complete surgical isolation of the heart in situ, as described by Bridges et al. [10], might enhance cell retention, allowing for more robust cell engraftment and myocardial regeneration.

ACKNOWLEDGMENT

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REFERENCES