

Cellular Cardiomyoplasty Using Autologous Satellite Cells: from Experimental to Clinical Study

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Abstract

Adult mammalian ventricular myocytes lack regenerative capability, consequently an injured heart is normally repaired by scar formation, hypertrophy of surviving myocytes, and hyperplasia of non-muscle cells. The possible existence of stem cells or progenitor cells for myocardium has been suggested recently, however it is clear that functionally significant myocardial regeneration has not been documented in diseased or injured heart. Contribution of other cells to the formation of ventricular myocytes appears to be negligible as evidenced by the consistent formation of scar after myocardial infarction. Satellite cells are adult stem cells responsible for growth, repair, and maintain homeostasis of skeletal muscle. We have been using autologous satellite cells for myocardial regeneration in dogs since 1989 and have applied this procedure to patients in 2001. Satellite cells have been successfully isolated, labeled, and implanted into injured heart with neomyocardial formation and functional improvement. Viable muscle cells with clear labeling are found in the infarct area after cell implantation. The labeled muscle cells have intercalated disks at cellular junctions. Significant improvements in contractile function are only observed in the animals that have successful engraftment after cell transplantation. Marked improvement in ejection fraction, myocardial perfusion, and local contractility are also found for patients after cellular cardiomyoplasty using autologous satellite cells. Most importantly, a heart failure patient after cellular cardiomyoplasty without conjunctival surgical procedure has recovered from congestive heart failure with significant improvement in myocardial perfusion, contractility, and metabolic activity at sites of cell implantation. Transplantation of satellite cells into injured heart can be a new treatment for myocardial infarction and heart failure.

Key words: cellular cardiomyoplasty, dog, heart attack, heart failure, man, satellite cells.

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Cardiovascular diseases remain the single largest cause of morbidity and mortality in the Western world and the United States [1, 2]. It is estimated that 12.6 million Americans alive today have coronary artery disease and more than one million people will suffer a heart attack every year. With longer life expectancy for the Western world, a further increase is anticipated for cardiovascular disease. Restoring blood flow, improving perfusion, reducing clinical symptoms, and augmenting ventricular function are the common treatments after myocardial infarction. Other than replacing the whole heart (cardiac transplantation) no standard clinical procedure is available to restore or regenerate the damaged myocardium following a heart attack. This report summarizes the experimental studies of cellular cardiomyoplasty using autologous satellite cells in dogs with our new observations. This paper

also includes our clinical studies using patients' own satellite cells implanted into the injured heart with or without simultaneous coronary artery bypass grafts.

Materials and Methods

Experimental animal

Mongrel dogs weighing 20 to 30 kg were purchased from a licensed vendor. The animals were housed in air-conditioned rooms with free access to food and water at all times. Humane care and proper analgesic, anesthetic, and tranquilizing drugs were provided when needed to all experimental animals. The "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Research Council in 1996 were followed. The proposed

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study was approved by the University Committee on Animal Care of East Tennessee State University.

After fasting and preoperative antibiotic treatment, each dog was anesthetized with sodium pentobarbital (15 mg/kg) and intubated with a cuffed endotracheal tube. After shaving the surgical sites and cleaning them with alcohol, the electrocardiogram was recorded using the PageWriter cardiograph and blood pressures were measured with Millar micro-tip pressure transducers (Millar Instruments, Inc., Houston, TX, USA). Anesthesia was maintained by 1% halothane and the surgical area was prepared with Betadine. The number of animals used for each study was detailed in the Results.

Patient selection

After obtaining approval from Department of Health (equal to FDA of USA) and Hospital Review Board (same as IRB of USA), patient selection and signed consent forms were established. Male patients (55 to 74 years of age) with recurrent exertion angina, abnormal ECG indicating history of myocardial infarction, and coronary angiography showing significant stenosis or blockage were recruited for the study. 2D-echocardiography, ^{99m}Tc -MIBI, and/or ^{18}F -deoxyglucose were used to determine the changes in ventricular function, myocardial perfusion, and metabolic activities before and after cellular cardiomyoplasty.

Isolation and culture of satellite cells

Under full anesthesia and sterile surgical conditions, a biopsy sample (15 ~ 20 g) from canine tibialis anterior muscle or a small sample (2 ~ 4 g) from right vastus lateralis muscle of the patients was obtained for cell isolation. The muscle sample was rinsed in 70% ethanol followed by Hank's basal salt solution without Ca^{++} and Mg^{++} but contained 1% penicillin-streptomycin and 1% amphotericin B. The tissue was minced (~ 1 mm³) before incubation in 10 volumes of enzyme solution in sterile 50 ml plastic tubes. The enzyme solution was made of buffered medium 199 containing 1% collagenase and 0.2% hyaluronidase filtered through a 0.2 μ filter and equilibrated with 95% O₂ : 5% CO₂. After 15 minutes of incubation at 37° C in a shaking water bath, the released satellite cells were harvested by pouring the solution through layers of sterile gauze. The remaining tissue was incubated in the enzyme solution at 37° C for another 15 minutes to complete the enzymatic liberation of satellite cells from muscle.

The isolated satellite cells were pelleted by centrifugation (650 x G for 10 min.) and washed with culture medium consisting of medium 199 with 10% fetal bovine serum and 1% antibiotic antimycotic solution (Sigma Chemical Co., St. Louis, MO). The viability of isolated cells was checked by trypan blue exclusion and the cell number was counted using a hemocytometer. At least half a million cells with better than 90% viability were commonly obtained from each gram of muscle. After proper dilution, 1 x 10⁶ cells were cultured with 8 ml of

medium in a 25 cm² culture flask. The isolated cells have a doubling time of 20 to 22 hours and can easily go through 20 cell cycles and still retain their proliferation and differentiation capabilities. The cells were subcultured every 3 to 4 days to maintain at low density for continual proliferation without differentiation. When forming multinucleated myotubes were desired, the satellite cells were cultured with medium 199 containing 2% horse serum and 1% antibiotic antimycotic solution until myotube formation to document myogenic capability.

Labeling of cultured satellite cells

To identify and follow the transplanted satellite cells at the site of injured heart, it is necessary to have long-term stable labeling of the cells with high intensity and specificity. Satellite cells enriched and proliferated by culture can be labeled with fluorescent microspheres (Polyscience Inc., Warrington, PA), ^3H -thymidine (Amersham Life Science Inc., Arlington Heights, IL), 5-bromo-2'-deoxyuridine (BrdU), 4'-6-diamidino-2-phenylindole (DAPI), dialkylcarbocyanine (DiI) or other membrane markers (Molecular Probes, Inc., Eugene, OR) as reported by us and other investigators [3-13]. The disadvantage of all these labeling procedures is the loss of labeling intensity as the cell divides (assuming equal division into daughter cells, 50% lower intensity for each cell cycle). Therefore, better labeling methods need be developed.

The lacZ gene which encodes β -galactosidase in *E. coli* and green fluorescent protein (GFP) have been used to label the cultured satellite cells. The mammalian reporter vectors lacZ and GFP (pCMV β) from Clontch Laboratory Inc. (Palo Alto, CA) and Lipofectamine from Gibco BRL (Gaithersburg, MD) have been purchased for the procedure. Very good labeling efficiency can be achieved using Lipofectamine, however AdenoLacZ and AdenoGFP from Quantum Biotechnologies (Montreal, Quebec, Canada) have outstanding labeling efficiency and the label can be maintained in cultured cells for more than eight weeks. Unfortunately, the AdenoGFP cannot be used for long-term *in vivo* study due to the strong inflammatory reactions and expression of viral proteins.

The humanized Renilla green fluorescent protein (hrGFP) using helper-free adeno-associate virus (AAV-hrGFP) purchased from Stratagene (La Jolla, CA) is used to label the cultured satellite cells. Since AAV can insert the DNA of interest into the host genome, long-term stable expression has been achieved using this method [14]. Use AAV to label the cells has the advantages of long-term expression, non-pathogenicity, inducing no immune response, physical stability, and the ability to transduce dividing and non-dividing cells [14-16]. The humanized GFP is selected to allow high expression of the fluorescence protein without the toxicity of eGFP [17, 18]. When AAV-hrGFP is used to label the cultured satellite cells, long-term high efficiency and high specificity of labeling has been achieved under *in vivo* condition. For clinical studies, the autologous satel-

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lite cells have been used without labeling to avoid any unexpected complication.

Ischemic injury and cell implantation in dog

Under proper anesthesia and sterile surgical preparation, the dog hearts were exposed through a mid-line sternotomy. The pericardium was opened and the edges attached to the chest wall to allow for good exposure of the left ventricle. After lidocaine (2 mg/kg) administration, the left anterior descending coronary artery and vein were ligated with 2-0 silk. The site of occlusion was just below the first branch of the coronary artery at about two thirds of the distance from the apex. Due to the rich collateral vessels in canine myocardium, obstruction of both arterial and venous flow was necessary to have a consistent ischemic injury. Reproducible myocardial infarction with low mortality (<5%) was achieved by this method.

After stabilization of cardiac function, the infarct zone was visually divided into 30 ~ 40 areas approximately 0.25 cm² each, before 0.1 mL of labeled autologous satellite cells (~ one million cells suspended in serum free medium 199) were injected at the center of each area. A 25 gauge hypodermic needle was used for the injection to avoid damage to the cells with smaller needles. After each injection a short pause (3 ~ 5 seconds) was given before removal of the needle while the needle hole was compressed by a finger with gentle pressure to avoid backflow and enhance cell retention. The control animals received serum free culture medium in the same manner. The sternotomy and incisions were closed in layers and animal was allowed to recover with analgesia provided as needed.

Measurement of hemodynamic functions in dog

The computerized Crystal Biotech VF-1 hemodynamic system (Data Science Int., St Paul, MN) was used to integrate the pressure, blood flow, contractility, and systolic wall thickening of each cardiac cycle. Millar micro-tip pressure transducers were positioned in the left ventricular cavity and aorta to determine pressures and dP/dt. The electrocardiogram was recorded using the PageWriter cardiograph. Digital sonomicrometer (Sonometrics Co., London, Canada) was used for real-time segment length and volume measurements. Ultrasound crystals were used for real-time determination of the long-axis (base of aorta to apex of heart), short-axis (ventral surface of mid-left ventricle to dorsal surface of mid-left ventricle), segment length across ischemic area, and wall thickness. The pressure-volume and pressure-length loops as well as percent thickening fraction were developed for evaluation of global and regional ventricular function.

Results

Morphologic observation of canine hearts after different treatments

Autologous satellite cells labeled with AAV-hrGFP were implanted into myocardial infarction of six dogs for regeneration of muscle cells. At six weeks after ischemic

injury, hearts without cell therapy (six dogs injected with serum free medium) showed significant amount of dense scar tissue with marked reduction in the thickness of left ventricular free wall (Figure 1). Only minimal amount of scar tissue was found for dogs at six weeks after myocardial infarction and cellular cardiomyoplasty. The morphology of cardiac sections from cell-implanted dogs was not different from normal heart (six dogs). The muscle cells developed from satellite cells have similar morphology and orientation as cardiac myocytes by light and electron microscopy [4, 5, 8, 9]. The proper orientation of muscle cells may be produced by adaptation to the mechanical stretch [19, 20]. In addition, the presence of intercalated disks and gap junctions between newly formed (from satellite cells) and host cardiac myocytes supported the likelihood of synchronized contraction for improvement of ventricular function [9].

Hemodynamic evaluations of canine heart

Using digital sonomicrometer and the VF-1 hemodynamic system, the pressure-volume loops of normal dogs and dogs subjected to myocardial infarction with and without satellite cell implantation at six week after the procedure were obtained (Figure 2). Although stroke work can be maintained in the hearts without cellular cardiomyoplasty, the ventricular diastolic pressure was significantly increased as compared to normal and cell implanted dogs (4 mmHg vs. 17 mmHg). The pressure-volume loops of the dogs without cell therapy showed a significant left tilt indicating abnormal left ventricular

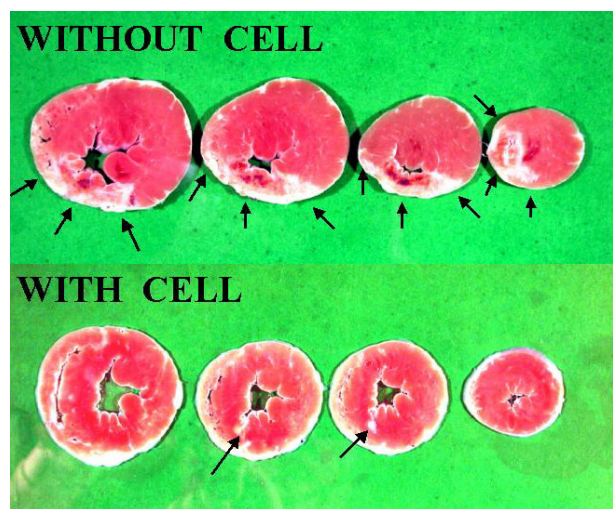


Figure 1. Slices of canine myocardium at six weeks after myocardial infarction with and without autologous satellite cell implantation. Thinning of ventricular wall with massive dense scar was observed from the heart without cell therapy. Cellular cardiomyoplasty restored the normal morphology of myocardium and drastically reduced scar tissue. Arrows pointed to the scar tissue. WITH CELL – implanted with autologous satellite cells after ischemic injury. WITHOUT CELL – injected with serum free culture medium after ischemic injury.

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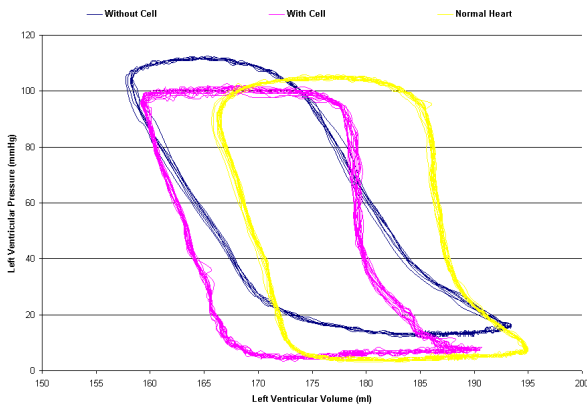


Figure 2. Pressure-volume loops of normal heart (yellow), ischemic heart with cell implantation (red), and ischemic heart without cell therapy (blue). At six weeks after ischemic injury, significant increase in left ventricular diastolic pressure and abnormal contractility were found for the heart without cell therapy. Cellular cardiomyoplasty restored left ventricular function.

contractile function. Dogs with ischemic injury and cellular cardiomyoplasty had similar pressure-volume loops as compared to normal animals. Transplantation of autologous satellite cells restored ventricular function after myocardial infarction.

Clinical outcomes

Three patients, all with a history of coronary heart disease, underwent coronary artery bypass grafting and implantation of autologous satellite cells. Satellite cells were isolated from biopsies of the right vastus lateralis muscle after enzymatic treatment. After proliferation in culture, satellite cells were harvested from culture flasks before washed with culture medium and suspended in 4 ml of serum free culture medium for implantation. This was done right after coronary artery bypass grafting while the heart still under hypothermic cardioplegia. The 4 ml cell suspension was divided into 30 ~ 40 doses and injected into the scar area (by palpation) and around the scar area. The needle holes were sealed with fibrin glue (Guangzhou Biotech Co., Guangzhou, China) before reversal of hypothermic cardiac arrest. Normally, the cell implantation procedure took less than five minutes for each patient.

After recovery, each patient was maintained at the intensive care unit for 72 ~ 96 hours with ECG monitoring before transferred to patient floor. All patients survived the procedure, without obvious arrhythmia, had an uneventful recovery, and were discharged from the hospital. At three to four months follow-up examination, increased left ventricular ejection fraction, decreased left ventricular diastolic diameter, and improved left ventricular wall thickness at the site of cell implantation were observed by 2D-echocardiography. Significant improvement in perfusion (^{99m}Tc -MIBI) and metabolic activity (^{18}F -deoxyglucose) were also found at the implantation

found at the implantation sites as compared to pre-treatment results. Occasional arrhythmias were detected while the patients were at intensive care unit, however treatment was not necessary. Arrhythmia was not observed during follow-up examination.

The fourth patient was a 73-year-old man with a history of myocardial infarction and hypertension for five years. He suffered heart failure (NYHA III-IV) with respiratory infection, urethra infection, and anemia. 2D-echocardiography indicated hypokinesia at septal, left ventricular anterior wall and apical regions. Left ventricular ejection fraction was 37.1%. Coronary angiograms demonstrated 60% stenosis in distal left main, 99% stenosis in proximal and distal left anterior descending coronary arteries. Both proximal and middle left circumflex coronary artery had 90% stenosis and diffuse stenosis of right coronary artery was found. Myocardial perfusion imaging using ^{99m}Tc -MIBI indicated defective perfusion of left ventricular apex, anterior wall, septal regions, and severe reduced perfusion of posterior inferior wall. Myocardial metabolic activities (determined by ^{18}F -deoxyglucose) also showed comparable reduction at the same areas of defective perfusion.

Coronary artery bypass grafting was planned for the patient with cellular cardiomyoplasty to be performed at the same time. After exposing the heart, left anterior descending, left circumflex, and right coronary arteries were all completely occluded and bypass procedure could not be completed. Autologous satellite cells were implanted without any complication and the patient had an uneventful recovery. During the first two months af-

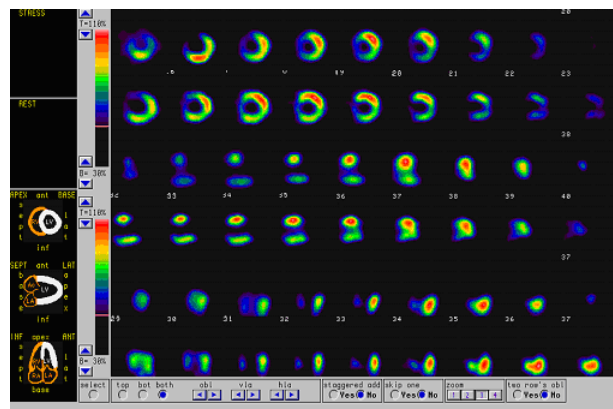


Figure 3. Myocardial perfusion image using ^{99m}Tc -MIBI. The perfusion images of the short-axis (top), vertical long-axis (middle), and horizontal long-axis (bottom) from the fourth patient before cellular cardiomyoplasty (upper line) and at five months after cellular cardiomyoplasty (lower line) were shown. A dramatic improvement in myocardial perfusion was observed at the left ventricular anterior wall (arrows) where autologous satellite cells were implanted. Contractility and metabolic activity showed similar improvement at sites of cell transplantation.

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ter cellular cardiomyoplasty, the patient remained in heart failure (NYHA III-IV) and required continual medical management. By the third month after operation, the patient's condition gradually improved and reached NYHA II. At five months after the procedure, significant improvement in left ventricular ejection fraction (37.1% to 48.6%) was observed (by 2D-echo). Left ventricular systolic diameter changed from 48mm (before) to 45 mm (after cellular cardiomyoplasty) and left ventricular anterior wall movement also improved. A dramatic improvement in myocardial perfusion using ^{99m}Tc -MIBI was observed (Figure 3) at five months after cell injection. Imaging with ^{18}F -deoxyglucose showed similar improvement in metabolic activity.

Discussion

Recent discoveries have demonstrated that in differentiated tissues of adult there are undifferentiated cells which are capable of self-renewal and proliferation resulting in production of progenitor and differentiated cells [21-24]. The fact that adult stem cells from one tissue appear to be capable of developing into cell types that are characteristic of other tissue is termed "adult stem cell plasticity" [24-29]. Satellite cells identified in skeletal muscle during 1961 [30], have been confirmed as myogenic precursor cells responsible for growth, repair, and adapting to physiologic demand of skeletal muscle [31-33]. Although satellite cells have been considered as monopotent stem cells, recent *in vivo* and *in vitro* studies clearly indicated that satellite cells from adult mammalian skeletal muscle are multipotent stem cells [34-37]. We have been using the autologous satellite cells from skeletal muscle for myocardial regeneration in dogs since 1989 [3]. Satellite cells have been successfully isolated, labeled, and implanted into injured heart with neomyocardial formation and function improvement [4-9]. Viable muscle cells with clear labeling were found in the infarct area after cell implantation. The labeled muscle cells showed intercalated disks at cellular junctions. Significant improvement in contractile function was only observed in the animals with successful cell transplantation [9].

Cell therapy has emerged as a strategy for the treatment of many human diseases. The aim of cell therapy is to replace, repair, or enhance the biological function of damaged tissue or organs. Satellite cells have been shown to regenerate skeletal muscle in all ages and species of mammals that have been investigated. Cellular cardiomyoplasty using autologous satellite cells offers several advantages: 1) they are autologous cells and immunorejection is not a concern, 2) they are not transformed cells and tumorigenesis may not occur, 3) they form gap junctions with the cardiac myocytes and arrhythmia has not been a complication, 4) they are not fetal tissue and will not engender ethical issue, 5) they are readily available from all patients and donor availability is not a limitation for autologous cells, and 6) they are highly resistant to ischemic and anoxic conditions and their survival after

transplantation is much better than other type of cells. Our experimental results and published data from other investigators clearly support the beneficial outcomes of satellite cell transplantation in injured heart. Early clinical applications offered highly encouraging results from others [38] and our own observations [39]. If these favorable early clinical observations can be confirmed by rigorous multi-center trials, cellular cardiomyoplasty using autologous satellite cells can be a standard procedure for patients suffering heart attack or heart failure.

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