Angiogenesis and Myogenesis in a Chronic Ischemic Heart.

Esha Ibrahim
East Tennessee State University

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Angiogenesis and Myogenesis in a Chronic Ischemic Heart

A thesis
presented to
the faculty of the Department of Physiology
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Biomedical Science

by
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August 2005

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Keywords: TMR, angiogenesis, myogenesis, myocardial ischemia, ventricular function
ABSTRACT

Angiogenesis and Myogenesis in a Chronic Ischemic Heart

by

Esha Ibrahim

Miniswine underwent procedures to evaluate treating chronic ischemia with the implantation of autologous satellite cells and laser transmyocardial revascularization (TMR). The objective was to combine two therapies to restore cardiac function. This experiment involved three surgical procedures: (1) placing a constrictor on the coronary artery; (2) producing channels and implanting cells; (3) obtaining samples. The swine were divided into groups: Group 1, Ischemia; Group 2, Ischemia + Laser TMR; Group 3, Ischemia + Laser TMR+ Cells; Group 4, Ischemia + Cells. Sonomicrometry and Millar pressure transducers were used to determine contractility, left ventricular pressure, and pressure-volume loops. There were no significant differences (p<0.05) among the hemodynamic data except for Group 4, which produced significantly lower output values. Morphological evaluation revealed a significantly reduced scar area in Group 3. Although there was a significant difference in scar area, the phenomena behind this improvement as compared to the unimproved hemodynamic function is not understood.
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Despite a significant advance in its prevention and treatment, coronary artery disease (CAD) remains the leading cause of death. According to the American Heart Association, nearly 13 million people in the United States suffer from some form of CAD. CAD is the most significant chronic condition and the leading cause of death for all segments of society. Conventional treatment for the disease includes medical therapies to reduce the risk factors, to reduce myocardial oxygen consumption, and to prevent progression of the disease; and interventional therapies are intended to restore the coronary artery blood flow either by angioplasty or surgery bypass (Braunwald 1997). These approaches, however, are limited by the development over time of native vessel narrowing and graft occlusions. There are a growing number of patients suffering from angina who, despite continued technical advances, cannot be treated by these conventional therapies. Accordingly, there is interest in exploring alternative forms of therapy to ameliorate angina symptoms and improve blood flow to ischemic myocardium. Transmyocardial revascularization is a newly-developed surgical treatment for patients with symptomatic end-stage coronary artery disease who are not candidates for conventional revascularization. Although it is still unresolved as to how laser transmyocardial revascularization achieves therapeutic effects, a growing amount of clinical and experimental evidence suggests that laser transmyocardial revascularization-induced angiogenesis plays an important role in this therapeutic approach.
Transmyocardial Revascularization (TMR)

Transmyocardial Revascularization (TMR) is a fairly new alternative treatment for patients with advanced coronary disease whose anatomy is not amenable to percutaneous coronary intervention or conventional coronary artery bypass graft surgery (CABG) (Allen et al. 2004). During TMR, small “channels,” or holes, are created in the heart via a laser device applied to the myocardial surface (See Figure 1).

![Laser Hand-piece in Contact with Myocardium (PLC Medical Systems, Milford, MA)](image)

The concept was based on the ability to achieve adequate myocardial perfusion by the creation of direct transmural channels through the ventricle. It is thought, as Figure 2 portrays, that the injury produced by the laser energy may result in the elaboration of vascular growth factors that stimulate angiogenesis (Horvath 2004a).
The application of TMR into clinical practice has been rapid and potentially driven by multiple factors. First, TMR represents one of the few existing treatment options for patients experiencing angina who do not qualify as candidates for traditional revascularization (Horvath 2004). In these patients, clinical trials have shown that TMR provided favorable symptomatic effect in many patients. Lastly, TMR requires little additional educational or technical training to
administer and commands professional fees similar to those required for traditional bypass surgery (Abo-Auda and Benza 2003).

It has been demonstrated that transmyocardial laser channels failed to acutely increase regional myocardial blood flow (Lutter and Yoshitake 1998). However, laser TMR is still being used to treat patients with diffuse coronary artery disease who are not amenable to conventional procedures (Samuels et al. 2004). The clinical experience with laser TMR indicates that angina is significantly relieved, perfusion and treadmill tolerance improved, and hospital admissions are decreased. The mechanism which facilitates reported laser TMR-related improvements remains something of a mystery.

**Angiogenesis Hypothesis.**

Several experimental studies have shown increases in vascularization which supports the angiogenesis hypothesis as an explanation for the unexplained phenomena behind Transmyocardial Revascularization. These studies have used a variety of methods to create the transmural channels: needles, variety of lasers, radio frequency energy, and growth factors. Nearly all devices investigated for TMR have succeeded in inducing angiogenesis in some form. Therefore, many investigators believe that angiogenesis could just be part of the normal healing process following myocardial injury.

Perfusion analysis has also provided evidence to support TMR. Because myocardial ischemia is primarily endocardial and typically not completely transmural, physiological redistribution of perfusion is more likely after TMR (Whittaker and Kloner 1997). Experimental results from procedures using swine evaluated at longer time intervals showed a significant
improvement in perfusion lending further support to the angiogenesis hypothesis. Human trials evaluating laser TMR have not shown perfusion improvements like those seen in animal studies, and currently there is no a sufficient explanation for this unfortunate difference.

**Cellular Cardiomyoplasty**

Cellular cardiomyoplasty is a procedure using cell transplantation to repair a damaged myocardium. Previous studies have shown evidence that syngeneic myoblast implantation following a myocardial infarction will elicit improved ventricular function (Jain et al. 2001). After years of investigation into the potential of using skeletal muscle cell grafting for cardiac repair, Menasche and his colleagues conducted the first clinical trial using skeletal myoblasts for MI repair eliciting encouraging results (Menasche et al. 2003).

**Satellite Cells: Identification and Significance**

Kao and colleagues have been using autologous satellite cell implants from the skeletal muscle of dogs even prior to 1989 (Kao et al. 1989). The satellite cells have been effectively harvested, labeled, and implanted into the injured heart to elicit new muscle formation and functional restoration (Jackson et al 2001). Other investigators have shown experimental results which support the implantation of autologous satellite cells from skeletal muscle as a potential approach for myocardial repair and functional improvement (Lash et al. 1957).

It is has been verified that unlike cardiac muscle, skeletal muscle maintains regenerative capabilities (Garrett and Best 1994). In 1961, Mauro first described the satellite cells of the skeletal muscle of a frog using electron microscopy. It is established that satellite cells play an
intricate part in skeletal muscle regeneration. The observance of the regenerative capabilities of satellite cells in skeletal muscle led researchers to consider the regenerative potential of myocardium (Marelli et al. 1992). This consideration led to the realization that cardiac myocytes are terminally differentiated cells that lose their ability to undergo cell division at an early age (Li et al. 1996).

Cellular cardiomyoplasty may prove to be more effective than coronary artery bypass graft and percutaneous coronary intervention therapies, which work to restore perfusion as well as to salvage dying muscle cells. Cellular cardiomyoplasty works to replace the cells that are already dead or lost (Rumyantsev 1977). Even though recent clinical trials have shown encouraging signs for this innovative technique, more research is critical in the area of cellular cardiomyoplasty to further evaluate efficacy and to attempt to understand the mechanism(s) underlying this improvement.

Satellite Cells: Muscle Repair

Over the years, it has become evident through the research of several investigators that satellite cell proliferation and induction are trauma-related (Schultz et al. 1978). This same work has also shown that satellite cells elicit new myofiber formation through a process analogous to muscle histogenesis in the embryo. Following injury, muscle goes through an unambiguous progression of healing phases. These healing phases include degeneration, inflammation, regeneration, and scar formation (Huard and Fu 2002). Degeneration and inflammation have been noted during the first few days after injury. Generally, regeneration begins approximately a week after injury and lasts up to a month at most, with peak performances noted at the two-week mark.
Fibrosis tends to begin between the 2\textsuperscript{nd} and 3\textsuperscript{rd} weeks following injury and continues to grow in size as time passes. After only a few cell divisions, satellite cell progeny start to fuse forming multinucleated myotubes, but the continued growth of the myoblasts, though largely dependent on the degree of injury, can persist for up to approximately 10 days (Snow 1978).

Satellite cells are located between the external lamina and sarcolemma of skeletal muscle fibers, which can be seen in Figure 3 (Hawke and Garry 2004). The presence of organelles such as ribosomes, rough endoplasmic reticulum, and golgi complexes implies the presence of metabolic activity in the satellite cells of immature muscle. This is in contrast to the low volume of such organelles found in adult muscle, which is indicative of a quiescent status. Investigators have previously shown that with the proper environmental signals, satellite cells enter into the cell cycle to provide the precursors necessary for new muscle formation in growth and repair (Jingbo and Liu 2004). It is thought that the disruption of the basal lamina and plasma membrane after injury releases the activated satellite cells.

![Figure 3 Satellite Cell Location (adapted from Hawke and Garry 2001)](image)
The regeneration of skeletal muscle has been conveniently separated into four divisions: (1) satellite-cell activation; (2) myoblast or precursor proliferation; (3) differentiation; (4) return to quiescence. Satellite-cell activation is the manner by which satellite cells exit G0 and enter the cell cycle (Hurme and Kallmo 1992). The second stage in skeletal muscle regeneration is the proliferation of satellite cells and myogenic precursor cells. Differentiation is the process whereby proliferating myoblasts derived from activated satellite cells and other myogenic precursors will withdraw from the cell cycle and either fuse to existing fibers in repair of damaged segments or to each other to form new fibers. Fusion events have been found to occur after cells exit mitosis and enter G1 (Hurme and Kallmo 1992). The ability of satellite cells to return to quiescence has been less thoroughly investigated than activation, proliferation, and regeneration. Under normal conditions the proportion of satellite cells that actually reside in G0 rather than in a lag phase in G1 is not known, as are many important aspects of satellite-cell quiescence. For example, it is uncertain whether the same satellite cells that originally respond to an activating stimulus will return to quiescence, or whether the satellite cell compartment is only repopulated by daughter cells (Shutlz and McCormick 1994).

Although the ability to identify satellite cells using histological methods will remain controversial until their characteristics and activities are further elucidated, it is recognized that the existence of multiple populations of myogenic precursor cells would allow muscle tissue to respond differentially to a particular stimulus, type of injury, or physiological demand, and thereby enable a highly controlled response (Rumyantsev 1991). The range of characteristics displayed by muscle precursor cells is most frequently made available through experimentation using the differences among cells isolated from muscle. For example, different lineages of muscle precursors
can be isolated from the variety of slow- and fast-twitch muscles in typical proportions, and each can differentiate to express distinct profiles of protein isoforms distinctive of slow and fast muscle. This justifies why the rectus abdominis skeletal muscle biopsy was used to isolate the satellite cells for this particular study involving myocardial regeneration. To sustain the heart’s workload, red muscle fibers which maintain slow fatigue characteristics are a practical selection for the repair of the cardiac muscle (Hawke and Garry 2001).

The basis for this procedure is to use the body’s natural repair process for skeletal muscle and extend this to the heart, because adult mammalian ventricular myocytes lack regenerative capability. Without intervention, scar formation is the final result of myocardial injury or damage. As long as scar is formed, complete regeneration of muscle tissue cannot occur, thus indicating that advances towards preventing muscle fibrosis as well as techniques to promote muscle regeneration must continue to be investigated.

The transplantation of satellite cells into an injured myocardium has resulted in improved myocardial function, but the mechanism of this improvement remains elusive (Kiortsis et al. 1989). Also, it still seems to be a topic of controversy concerning whether transplanted satellite cells differentiate into modified skeletal muscle fibers or into actual cardiac muscle cells, as once hypothesized, or if what has looked like transdifferentiation is actually cell fusion or contamination of the biopsy sample with unfamiliar progenitor cells and/or pluripotent cells that are stimulated to form new progenitor cells of a different tissue lineage (Young and Black 2004).
Rationale for Thesis

A significant amount of data has suggested that providing ischemic myocardium with angiogenesis through laser TMR leads to neovascularization and to myocardial protection in the remodeling process after chronic myocardial ischemia. Clinical observation and data have also demonstrated that a patient’s angina symptoms can be relieved within days after laser TMR treatment. The neovascularization developed through angiogenesis, however, does not occur immediately or even a few days after various laser, mechanical, or even pharmacological stimulation. It takes at least weeks to develop new vessels in the ischemic myocardium after the expression angiogenic factors. Does laser TMR provide an increase in regional perfusion following chronic ischemia? Is there a significant improvement observed in cardiac function and scar formation following elicitation of cellular cardiomyoplasty and laser TMR therapies? Is there a greater improvement seen in global and regional contractility using two unconventional therapies in combination as compared to each therapy treating chronic myocardial ischemia individually? Based on these inquiries and relevant clinical research, evaluating the effectiveness of combining two unconventional therapies is the primary objective of this thesis.
CHAPTER 2
MATERIALS AND METHODS

Materials

Animals

Thirty miniswine, male and female, weighing 25-30kg were obtained from Harlan-Sinclair, Indianapolis, IN.

Satellite Cells

Satellite cells were harvested from the rectus abdominus muscle at the lower chest portion of the miniswine after the completion of the first sternotomy procedure. After isolating the cells and allowing them to plate on the flasks, the cells were then transfected and labeled using humanized green fluorescent protein from Renilla reniformis. (Stratagene Cloning Systems, La Jolla, CA).

CO2 Heart Laser

Transmyocardial Laser Revascularization (TLMR) was performed using an 800-W CO2 laser (The Heart Laser, PLC Medical Systems, Milford, MA).

Ameroid Constrictor

An ameroid constrictor was used to simulate the gradual constriction of the artery over time as coronary artery disease, inducing chronic ischemic damage (2.0mm ameroid constrictor, Research Instruments, SW, Escondido, CA).
Gortex Surgical Membrane

A semipermeable membrane made from expanded PolyTetraFluoroEthylene (PTFE) (Gore, Flagstaff, AZ).

Media

* M-199 containing 1% antimycotic antibiotic solution (contains penicillin, streptomycin, and amphotericin B) purchased from Sigma Chemical Co., (St. Louis, MO) and 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO).

* Hank’s balanced salt solution lacking calcium and magnesium (Sigma)

Enzymes

Hyaluronidase (Sigma Chemical Co., St. Louis, MO)

Collagenase (Sigma Chemical Co., St. Louis, MO)

Trypsin containing EDTA (ATCC, Manassas, VA)

Microspheres

Non-radioactive injectable, colored ultraspheres formulated as 10 million spheres/ml suspended in saline with Tween 80 & Thimerosal(E-Z Trac, Irvine CA).

Syringe Pump

An infusion/ withdrawal pump, model # 906 (Harvard Bioscience Co., Holliston, MA), was used to obtain a reference blood sample for regional perfusion calculations with the microspheres.
Methods

Study Design

A chronic ischemia myocardium swine model with an ameroid constrictor on the left anterior descending coronary artery was designed for laser TMR and cellular cardiomyoplasty to induce angiogenesis and myogenesis in order to improve regional blood flow along with overall cardiac function in ischemic myocardium. The reasons for choosing this particular model were as follows:

1. The larger animal model makes it technically more feasible to evaluate the functional study and to measure the regional blood flow.

2. Similar to humans, swine myocardium has little native collateral circulation
Experiment Time Line for In Vivo Study

9 weeks

Ischemia Group

<table>
<thead>
<tr>
<th>Baseline</th>
<th>TMLR treatment</th>
<th>Termination &amp; heart extraction</th>
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<tr>
<td>Placement of LAD occluder-</td>
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3 weeks 6 weeks

Ischemia + Laser Group

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3 weeks 6 weeks

Ischemia + Laser + Cells Group

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3 weeks 6 weeks

Ischemia + Cells Group

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<td>Placement of LAD occluder-</td>
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**Animals**

Miniswine weighing 25 to 30 kg were purchased for the proposed study. The animals were housed in air-conditioned rooms with controlled light and dark cycles and given free access to food and water at all times except when prepared for or under surgical procedures. After fasting overnight and receiving preoperative treatment, each pig was anesthetized with 8mg/kg telazol and 1mg/kg of xylazine following ear vein cannulation. The animals were intubated with a cuffed endotracheal tube following an injection of atropine (0.05mg/kg). The surgical sites were shaved followed by a thorough cleaning with betadine. A baseline electrocardiogram (EKG) was obtained using a 12-lead Hewlett Packard machine. Also, the EKG was monitored throughout the procedure with leg leads. Millar micro-tip pressure transducers were used to measure blood pressures. Anesthesia was maintained with 1% to 2% halothane. Humane care and proper analgesic, anesthetic, and tranquilizing drugs were provided to all experimental animals as needed. The “Principles of Laboratory Animal Care“ and the “Guide for the care and use of Laboratory Animals” were followed. The proposed study was approved by the Animal Care and Use Committee of East Tennessee State University. Thirty experimental animals were used for myocardial regeneration using cellular cardiomyoplasty and Laser Transmyocardial Revascularization. The animals were categorized into four groups: Group 1, Ischemia; Group 2, Ischemia + Laser TMR; Group 3, Ischemia + Laser TMR + Cells; Group 4, Ischemia + Cells. The same anesthetic regimen was used for each of the three different surgical procedures. Continuous electrocardiographic and blood pressure monitoring was used throughout each procedure to ensure a stable cardiac rhythm and pressure status. Antibiotics were administered postoperatively by a veterinary technician. Also, pain medications were administered
intramuscularly following surgery as needed or until the normal activity level of the animal was resumed.

**Autologous Stem Cell Isolation, Cultivation, and Labeling**

Skeletal muscle samples were obtained from the rectus abdominis (skeletal) muscle at the chest wall of the experimental animals during the first surgical procedure. The muscle was rinsed with 70% ethanol followed by three rinses in Hank’s balanced salt solution lacking calcium and magnesium but containing 1% antimycotic antibiotic solution (5ml of antibiotic added to 500ml M-199). The tissue was minced and then incubated with 20 mL of enzyme solution (1% collagenase and 0.2% hyaluronidase). After 10 minutes of incubation at 37°C, the satellite cells were harvested by pouring the solution through layers of sterile gauze into a sterile 50 mL centrifuge tube and pelleted by centrifugation. The remaining tissue was incubated for 5 more minutes in enzyme solution to complete the release of satellite cells from the muscle. The isolated satellite cells were washed three times with medium-199 containing serum (10% fetal bovine serum; 50ml added to 500ml of M-199) and 1% antibiotic antimycotic solution by centrifugation and then resuspended. The cell number was counted using a hemocytometer. After proper dilution, 1 X 10^6 cells were cultured with 8 mL of proliferation medium in a 25cm² flask. The isolated cells have a doubling time of approximately 22 hours. They can easily go through 20 cell cycles and still retain their proliferation and differentiation properties. Cells were generally labeled at 5-6 days after isolation when the cells were steadily growing and had adhered to the flask. The cells were labeled via a viral transfection method using humanized green fluorescent
protein from Renilla reniformis. This humanized green fluorescent protein was added (0.8mL per 25cm² flask) to the cells after removing the culture media. After a 3-hour incubation, enough media to cover the bottom of the culture flask was supplied to the cells. Twenty-four hours later the media was again replaced with fresh culture media. The fluorescence could be observed in the cells as early as 48 hours post transfection using a fluorescent microscope with a FITC filter.

Myocardial Infarction

During the first surgical procedure following skeletal muscle biopsy of the chest wall and baseline recording measurements, the left anterior descending coronary artery was isolated and the ameroid constrictor was placed around the vessel.

Cell Preparation for Implantation

After discarding the culture media, the satellite cells were rinsed with Hank’s Balanced Salt Solution containing 1% antibiotic to removed any remaining serum that would prevent the cells from detaching. Next, the cells were incubated in a 5:1 dilution of plain Hank’s Salt Solution containing Trypsin and ETDA. The detachment of the cells from the flask was observed via a light microscope and the cells were pelleted using centrifugation for five minutes and then were resuspended in 4mL of plain M-199 culture media. The cell number was assessed using a hemocytometer. Cells were kept sterile and when needed were poured into a 5mL syringe and then injected into the injured myocardium. A representative aliquot of the cells were saved and allowed to continue their proliferation in a 25cm² flask to observe the growth of mononucleated satellite cells into multinucleated myotubes. The reserved samples were fixed with buffered formaline and
kept in phosphate buffered saline until pictures could be captured using the fluorescent microscope with a FITC filter to observe the fluorescently labeled myotubes.

**Laser Transmyocardial Revascularization (TMR)**

At 3 weeks after the initial operation while under sterile surgical preparation and following hemodynamic evaluation, the heart was exposed through a sternotomy. Laser TMR was performed with an 800-W CO2 laser (The Heart Laser, PLC Medcial Systems, Milford, MA) using a single 20-J pulse timed to the R wave of the EKG; 20 channels were created at 1 cm intervals for the ischemic myocardium. After laser TMR and the establishment of hemostasis, 0.1mL of GFP labeled autologous satellite cells were injected at the center of each channel using a syringe with a 25G hypodermic needle. The laser TMR control animals were subjected to the same treatment but were injected with serum free culture medium. The carbon dioxide laser is timed from the R-wave of the EKG to allow a successful channel to be made with the laser instrument when the ventricle is blood-filled. The two groups of miniswine not receiving satellite cell injections either received laser treatment followed by injections of plain culture medium into the channels or no laser treatment and no injection. There also was a group of swine that received satellite cell injections into the ischemic myocardium without experiencing the heart laser treatment. After closing the sternum and incision, the animals recovered in cages and received the same post-operative care and medication as at the time of the muscle biopsy and ameroid constrictor placement.
Hemodynamic Evaluation

The aortic and venous pressures were determined using Millar pressure transducers (Millar Instruments, Inc., Houston, TX). The EKGs were recorded during each surgical procedure using a Hewlitt Packard electrocardiogram 12-lead machine. The blood flow, contractility, systolic wall thickening, pressures, and EKGs were integrated using the computerized VF-1 hemodynamic system (Data Science Inc., St Paul, MN) and the Digital Sonomicrometer (Sonometrics Co., London, Canada). Ultrasonic crystals were placed at the long axis of the heart (crystals 1-2), the short axis of the heart (crystals 3-4), across the depth of the left ventricular free wall (crystals 3-5), and at the border of the ischemic area (crystals 6-7) (See Figure 4). The cardiac output, pressure length loops, pressure volume loops, ejection fraction, and stroke work were used to determine the global and local contractile functions.

Figure 4. Depicts sternotomy procedure of swine with crystals, constrictor, and pressure transducers labeled. 1. aorta  2. apex  3. anterior  4. posterior (under the heart)  5. myocardial wall thickness  6. right infarct area  7. left infarct area ; LVP= left ventricular pressure transducer, AP= arterial pressure transducer, C= ameroid constrictor
Global Contractility

A way to assess the pumping function of a particular heart is to evaluate the work loops for that heart. The work loop is derived for each heart beat in real time and is a graphic relationship between the changes in left ventricular pressure compared to the left ventricular volume during a single heart beat (See Figure 5). The P-V loops were generated by simultaneously recording ventricular pressure and volume in the working heart. Ventricular pressure was obtained using an intra-ventricular pressure catheter. Left ventricular volume was approximated by employing the equation for an ellipsoid, which is considered a well-established method. By using a pair of crystals, one pair measuring the long axis (from the base to the apex) and the other pair measuring the short axis (on the equatorial posterior and anterior LV free wall), along with application of the ellipsoidal equation, the left ventricular volume was calculated per animal per cardiac cycle.

![Pressure-Volume Loop](image)

**Figure 5.** Pressure-Volume Loop illustration which allows LV function assessment. The left ventricular volume (LV V) comprises the x-axis and the left ventricular pressure (LVP) comprises the y-axis. a. represents ventricular filling  b. represents isovolumetric contraction c. represents ventricular ejection d. represents isovolumetric relaxation SV= stroke volume, ESPVR= end-systolic pressure volume relationship, EDPVR= end-diastolic pressure volume relationship, ESV= end-systolic volume, EDV= end-diastolic volume (Richard E. Klabunde, www.cvphysiology.com, 2005)
Generating pressure-volume loops allows the assessment of myocardial compliance or commonly referred to as Emax. The Emax is the slope of the regression line that fits the end-systolic points on a pressure-volume loop after temporary occlusion of vena cava. Cardiosoft determines these points by identifying the location in the cardiac cycle where the ratio of instantaneous pressure to instantaneous volume, minus the volume axis intercept, is greatest. These points are located for all beats selected and used to calculate E-max.

Cardiac Output Cardiac output is basically a flow rate that is directly related to the heart rate and stroke volume. Cardiac output is calculated on a beat-to-beat basis and the period is defined from end diastole to end systole. Cardiac output is a function of the end-diastolic volume. Cardiac output is expressed as the following equation:

\[ \text{CO} = \text{SV} \times \text{HR} \]

Ejection Fraction Ejection Fraction is the fraction of the end-diastolic volume ejected in each stroke. Ejection Fraction is expressed as the following equation:

\[ \text{EF} = \frac{\text{SV}}{\text{EDV}} \]

Regional Contractility

As the heart contracts and relaxes, the cardiac muscle undergoes various changes in wall thickness and segmental shortening. The magnitude of these changes can be affected by a variety of physiological and pathological processes. Therefore, quantification of these changes provided valuable information concerning functionality and contractility. Segment shortening percentages
express the changes in segment length between the minimum distance and end diastole. Segment shortening is expressed as the following equation:

\[ SS\% = \frac{[SL(ED)- SL(ES)]}{SL (ED)} \]

**Regional Perfusion**

The colored microspheres prepared by E-Z TRAC were crosslinked polystyrene-divinylbenzene microspheres. The method of animal preparation and microsphere injection with the E-Z TRAC colored microspheres were the same as that followed with radioactive microspheres in blood flow studies. To measure regional myocardial blood flow, the microspheres were injected into the left ventricle of the experimental animal during each surgical procedure just before chest closure. A line was placed into the left or right internal mammary artery, then a 2ml solution was injected into the heart (1.4 ml sucrose solution and 0.6 ml microspheres), followed by a reference blood sample collection at a rate of 15 ml/min for one minute with a syringe pump. The reference blood sample was placed into a 50ml centrifuge tube with 0.1ml of heparin, labeled with the pig identification number and date, and then blood reagent solution from E-Z TRAC was added (see Figure 6). Once injected, the E-Z TRAC colored microspheres then mixed with the arterial blood, distributed to the tissue, and ultimately lodged in the microvasculature. The number of microspheres in the myocardial tissue of interest and in reference blood sample were determined by following an extraction procedure using reagents and instructions supplied by E-Z TRAC. After completing the process to retrieve the microspheres from the reference blood sample or from the myocardial tissue, the spheres were counted using a hemocytometer.
Statistical Analysis

A one-way ANOVA statistical test from Excel was used to compare the means among the groups. Also, a non-parametric analysis, Kruskal-Wallis, with minitab software was used. A t-test was used to assess the differences of the calculated values for each group of hearts at each time point as compared to the group receiving only a coronary occlusion termed as Ischemia Group. P<0.05 was considered significant in all statistical tests. This method of statistical
analysis was used to assess statistical significance for all cardiac function values evaluated in this project.

**Heart Harvest**

The animals were deeply anesthetized with halothane. The chest cavity was opened exposing the heart. The heart was removed from the chest cavity, rinsed with 0.9% saline solution and placed in a labeled, sealed bag to be frozen in order to section. Additionally, the animals were heparanized prior to organ harvest, which inhibited blood clotting and enabled better tissue cleanup.

**Heart Sectioning**

The heart was sectioned into 0.2 cm slices using a toastmaster platinum electric food slicer. The tissue was then placed in iced buffered formaline. The heart tissue sections were then processed, paraffin embedded, and sectioned by James H. Quillen’s pathology department’s histology technician.
CHAPTER 3

RESULTS

Isolation of Satellite Cells from the Skeletal Muscle of Miniswine

Light microscopy allowed morphological evaluation of the satellite cells. Figure 7 illustrates that in this experiment, satellite cell isolation, proliferation, and labeling were successfully completed. Panel d of Figure 7 shows the formation of the satellite cells into multinucleated myotubes and the presence of the fluorescence in the myotubes as well.

Figure 7. Light Microscopy Pictures of Swine satellite Cells a) early culture of passage 1 cells in 20% fetal bovine serum (FBS) b) early culture of cells with green fluorescent protein (GFP) label c) formation of multinucleated myotubes from mononucleated cells d) multinucleated myotubes with GFP label.

Cell Counts

No correlation could be made between number of injected cells and functional improvement. However, this element of cellular cardiomyoplasty remains elusive despite the avid interest in the question that remains: Is there a minimal or maximal cell implantation number
necessary to have beneficial effects? In addition, the range of cells implanted in this experiment is too close (1.3 X 10^6 to 6.5 X 10^6 cells) to have a meaningful evaluation for cell number and functional recovery.

**Pathological Evaluations**

Sectioning the pig hearts allowed us to visualize the scar portion versus the area of viable myocardium. Figure 8 illustrates the method used in sectioning the hearts. Pictures were taken of these sections using a digital camera which allowed transference of this information into an imaging program (See Figure 9). The imaging software made the scar area percentage calculation possible through freehand visualization and interpretation. The chart (See Figure 10) shows the percentages of scar area per group. A significant decrease in the scar area was found for the ischemia + laser + cell group.
Figure 8. Illustration of how the swine hearts were sectioned: C designated control region and (i) designated the ischemic region of the myocardium assigning two (1 & 3) of the tissue sections for digestion for microsphere counts and two to be frozen (2 & 4) to be used for pathological evaluation.

Figure 9. Representative Heart Sections from each Animal Group A) Ischemia B) Ischemia + Laser C) Ischemia + Laser + Cells D) Ischemia + Cells. Morphological evaluation of these heart sections allows for the observation and comparison of the left ventricle (LV) scar area.
Electrocardiograms

During the cardiac cycle, the electrical event precedes the mechanical. The electrocardiogram (ECG or EKG) is the standard clinical tool used to evaluate the electrical activity of the heart non-invasively. It is a graphic record of the sum of the cardiac action potentials from different regions of the heart that can be picked up by recording electrodes from the surface of the body as potential differences or voltage differences. These differences are
recordable because of the timing and sequence of depolarization and repolarization of the heart. Assessment of the height of various waveforms and the duration of the waveforms and their various components allows insight into the normalcy or pathology of the electrical nature of the heart. The electrocardiogram shown below (Figure 11) illustrates that what was seen in the pig recordings at baseline (a normal sinus rhythm) and what was observed following LAD occlusion (ST segment elevation indicative of myocardial injury).

Figure 11 Electrocardiogram showing lead II in both panels: A) Normal Sinus Rhythm of minipig heart B) ST elevation & fibrillation (6th beat) after ischemic injury
Hemodynamic Evaluations

The contractility and functionality of these hearts were evaluated in each pig at two distinct but consistent time points. The measurements of the heart that enable the evaluation of the function each heart were recorded using ultrasonic crystals attached at seven points on the heart (discussed in more detail in the Materials and Methods section). These measurements were obtained at a baseline value, before any treatment was elicited, and again just before termination, nine weeks after the initial “normal” recordings. In comparing the different components of function, the baseline value for each animal was designated as 100%. For each animal cardiac output, segment-shortening, ejection fraction, and E-max were evaluated during each of the surgeries. Measurements of hemodynamic parameters using 1-way ANOVA, t-tests, and Kruskal-Wallis comparisons revealed no statistically significant differences (P=ns).

Cardiac Output

Cardiac Output is directly related to the stroke volume and heart rate. We evaluated cardiac output for each group and expressed the values by assigning the recorded baseline measurement as 100% as compared to the value obtained during the termination procedure. As expected, two of the treatment groups, ischemia + laser and ischemia + laser + cells, showed clear increase, though not statistically significant, in cardiac output as compared to the control ischemic group. However, an unexpected result was the low output percentage values of the group receiving ischemia + cells. The graph (Figure 12) represents the cardiac output percentages for each of the four categories of animals. There was no significant difference observed in cardiac output among the experimental groups, though there was a general observable trend that would
require further research in order to determine whether the differences would reveal significance using larger population samples as well as evaluating the animals progression at longer time points.

Figure 12. Graphical Representation of Cardiac Output: SEM indicated by black bar lines; Significance was assessed using 1-way ANOVA. No significant statistical difference was found among the groups (p=ns).

Ejection Fraction

Ejection Fraction is another assessment of global contractility and it is the ratio of the stroke volume to the end-diastolic volume. There is no significant difference among the groups (Figure 13). These values differ only modestly and revealed no significant functional difference in the ejection fraction percentages using the baseline values as 100% across the groups.
Figure 13. Graphical Representation of Ejection Fraction: SEM indicated by black bar lines. Significance was assessed using 1-way ANOVA where no statistical significance was found (p=ns).

**Segment Shortening**

Percent myocardial shortening in the LAD region is demonstrated below in Figure 14. The SS% of the remote myocardium did not reveal differences among the first three groups. However, there was a significant reduction of the regional contractility in the ischemia + cells group as compared to the ischemia + laser group (p<.05). This was attributed to the inflammatory response as a result of the Gortex membrane used as a synthetic pericardium in an effort to reduce adhesion formation seen in greater than 50% of the pigs from this particular group. Also, there
was noticeable reduction in regional contractility of the other two groups as compared to the ischemia + laser group.

**Segment Shortening (6-7)**

![Graphical Representation of Segment Shortening](image)

Figure 14. Graphical Representation of Segment Shortening: SEM indicated by black bar lines denotes significant difference between the ischemia + cells group as compared to ischemia + laser group (p<0.05). Significance was assessed using 1-way ANOVA followed by a t-test comparison.

**Pressure-Volume Loops**

A single left ventricular cycle of contraction, ejection, relaxation, and refilling can be visualized using a pressure-volume loop. The pressure volume loops were generated by simultaneously measuring the left ventricle using a pressure catheter and by evaluating the volume of the left ventricle using the ultrasonic crystals. Several factors can elicit a alteration in the pressure-volume loop from its baseline (normal) form. The stroke volume is the volume of blood
ejected from the ventricle on each heart beat and can be estimated using the pressure-volume loops by subtracting the end-systolic volume from the end-diastolic volume. There was no significant difference in stroke volume values among the groups (See Figure 15). The following chart (See Table 1) lists the stroke volume (SV) values calculated for each animal of each animal group category. The highlighted selection from each group was chosen using the average value obtained for each animal group. Each highlighted animal was then used to create the representative loops (See Figures 16-19) from each categorical group to observe what was occurring during the cycle of the heart through the use of pressure-volume loops.

Figure 15. Graphical Representation of Stroke Volume: SEM indicated by black bar lines denotes significant difference between the ischemia + cells group as compared to ischemia + laser group (p<0.05). Significance was assessed using 1-way ANOVA, followed by t-test comparison.
Table 1. Stroke Volume Values and Averages per Animal and per Animal Group

The stroke volume values for each animal of each group and mean values for each group at baseline and at sacrifice time points are listed.

### Stroke Volume

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Laser</th>
<th>Laser + Cells</th>
<th>Cells</th>
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<td>Baseline</td>
<td>Sacrifice</td>
<td>Baseline</td>
<td>Sacrifice</td>
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<td>30</td>
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<td>20</td>
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<td><strong>3028</strong></td>
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<td>30</td>
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<td>25.525</td>
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<tr>
<td><strong>3019</strong></td>
<td>25.4</td>
<td>17</td>
<td><strong>Mean=</strong></td>
<td>26.225</td>
</tr>
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</table>
Figure 16. Representative p-v loops from the ischemia only animal group at baseline and sacrifice time points. From Baseline to sacrifice, these Pressure-Volume (PV) loops show a marked increase in the left ventricular volume, a stroke volume of approximate equal proportions and no real change in the overall left ventricular pressure. However, both the end-diastolic and end-systolic volume increased a substantial amount.

Figure 17. Representative p-v loops from the ischemia + laser animal group at baseline and sacrifice time points. In the representative loops from the Ischemia + Laser group, there was no great change in the overall left ventricular pressure or in the left ventricular volume from baseline to sacrifice. The end-systolic volume and end-diastolic volume did decrease slightly but the stroke volume remained relatively the same.
Figure 18. Representative p-v loops from the ischemia + laser + cells animal group at baseline and sacrifice time points. From baseline to sacrifice, the ischemia + laser + cells representative loops illustrated a rather large shift in left ventricular volume but the left ventricular pressure remained relatively the same. The end-systolic and end-diastolic volumes dropped an observable amount with only a slightly noticeable decrease in stroke volume.

Figure 19. Representative p-v loops from the ischemia + cells animal group at baseline and sacrifice time points. In the ischemia + cells representative loops, there were observable increases in left ventricular volume and pressure. The end-systolic and end-diastolic values both increased but the overall stroke volume and stroke work decreased relatively.
An increase in stroke volume is the result of an increase in end-diastolic volume, which is the result of increased venous return (effects of changes in preload). The increase in stroke volume is reflected in increased width of the pressure-volume (p-v) loop. The p-v loops that indicated a decrease in stroke volume reflected the change with a decrease in the width of the p-v loops. The decrease in stroke volume results in a decrease in end-systolic volume. In general, all the loops show a decrease, in some cases very slight, in pressure from the normal or baseline pressure-volume loop which can be viewed as a loss in height of the loop and correlates with decreased contractility from baseline for each of the categories.

As mentioned, the slope of the line through the points at the end of the systole represents the end-systolic pressure-volume relation (ESPVR) and is often used as an index of contractility. Therefore, the slope remains unchanged without any accompanying changes in contractility even with changes in preload or afterload. The slope of the line of the ESPVR for each loop for each subject were compared and averaged across the groups using the baseline value as normal, or 100% (See Table 2). Again, there were no statistically significant differences among the groups that were evaluated using 1-way ANOVA statistical analysis tests, accepting significance as a p-value less than 0.05.
Table 2. Load Variables and Averages per Animal and per Animal Group: Emax, EDPVR (End-diastolic Pressure-Volume Relationship), & PRSW (Preload Recruitable Stroke Work) for each group and the mean value per group. A 1-way ANOVA comparison of the means among the groups reveals no significance (p=ns).

Load Variables (values @ sacrifice)

<table>
<thead>
<tr>
<th></th>
<th>Emax</th>
<th>EDPVR</th>
<th>PRSW</th>
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<tbody>
<tr>
<td><strong>Ischemia</strong></td>
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<td></td>
<td></td>
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<td>0.192</td>
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</tr>
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<td><strong>Mean</strong></td>
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<td></td>
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</tr>
<tr>
<td><strong>Mean</strong></td>
<td>0.846</td>
<td>0.498</td>
<td>0.545</td>
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</tbody>
</table>
Regional Perfusion

The microspheres were used to compare regional perfusion as a result of angiogenesis or lack thereof in each of the randomly assigned groups (See Table 3). There were only very slight differences in the flow per gram per milliliter between the groups.

Table 3. Microsphere Counts to Measure Regional Perfusion. Represents microsphere counts from the ischemic region of the myocardium per animal group category: LAD= left anterior descending coronary artery; N= number of animals per group for regional perfusion analysis

<table>
<thead>
<tr>
<th>Regional Myocardial blood flow (ml/100g/min)</th>
<th>9 weeks post ameroid constrictor placement</th>
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<tr>
<td></td>
<td>Ischemia</td>
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<td>LAD region</td>
<td>44</td>
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<tr>
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</table>
CHAPTER 4
DISCUSSION

Miniswine satellite cells were harvested and labeled successfully. Pathological evaluations revealed significant scar area reductions in the cell + laser TMR and the cell groups. This exciting result was puzzling because the hemodynamic analysis, which evaluated cardiac function, resulted in no supporting functional improvement, specifically in the laser TMR + cells group. However, since this loop was only a representative and did not include the entire group, its seemed overreaching to use it to make any concrete claims, especially when considering the results of the other functional parameters. In the laser TMR + cells group, the left ventricular volume was decreased while maintaining cardiac output. The ischemia representative loop showed an increase in left ventricular volume with a decreased cardiac output which together are indicative of a failing heart. For all of the animal groups a 50% reduction was observed in the ejection fraction values, which is not atypical following myocardial ischemia, however, it would seem worthwhile to evaluate the ejection fraction at greater time intervals.

Autologous stem cell therapy has been shown to induce regeneration of viable myocardium (Chiu et al. 1995). The mechanism behind this phenomena has yet to be elucidated (Jingbo et al. 2004). According to the American Heart Association, cardiovascular disease (CD) remains the number one killer of men and women in the United States. It was hypothesized that a combination therapy using transmyocardial laser revascularization in addition to cellular cardiomyoplasty would elicit angiogenesis and myogenesis in a chronic ischemic heart. The aim of our study was to evaluate the effectiveness of combining these two therapeutic techniques (Horvath et al. 2001).
Laser TMR is FDA approved and has been used to treat patients suffering from chest pain who do not otherwise qualify as participants in other traditional revascularization techniques. However, previous studies in the CD literature have shown that autologous stem cell implantation following ischemic injury results in improved ventricular function. (Jain et al.) However, whether or not the implantation of satellite cells improves cardiac function has yet to be confirmed (Wozniak et al. 2004). Based on these results, it is impossible to confirm or reject this implication and therefore, no further insight has been gained into the mechanism behind the regeneration phenomena. The lack of biochemical markers for satellite cells has made isolating a pure population of these cells and the identification of these cells after implantation quite challenging. For many years, the goal of advancing myocardial neovascularization has been studied in hopes of providing an alternative therapy to those patients who cannot undergo conventional revascularization (Yoon, Kao and Magovern 1995). Still, many questions surrounding this topic remain: If there is an improvement seen following satellite cell implantation, what is the mechanism? Once these cells are implanted into the injured myocardium, where do they go or what do they become? Is there a best way to administer the implantation of the cells and is there a necessary number of cells needed for implantation?

Over approximately the last 40 years, numerous experimental studies have been performed to investigate the revascularization potential of transmyocardial revascularization and the possible working mechanism underlying the observed clinical improvement in angina pectoris after this treatment (Menno et al 2002). An acceptable explanation for this clinically observed relief has not been found. Over the years, a number of hypotheses have been used to explain the effectiveness seen with transmyocardial revascularization therapy. The “patent channel”
philosophy has been somewhat recently dismissed as a possibility and has left “angiogenesis” and “denervation” hypotheses competing for the “final answer.” However, since supportive evidence has been presented which gives credit to both proposed mechanisms, it can be deduced that perhaps both play a role in this phenomena. It is apparent that more clinical studies and further research must be conducted in order to obtain a clearer understanding of the mechanism behind laser TMR as well as to optimize the delivery of the therapy in general.

Implantation of autologous skeletal myoblasts (satellite cells) as an approach for myocardial repair and functional improvement has been confirmed by other researchers (Bischoff 1994). The benefits of employing autologous satellite cells are further supported by more recent publication and encouraging preliminary clinical evaluation by Kao and colleagues as well as others (Jackson et al 2001). If favorable observations can be confirmed by rigorous clinical trials, cellular cardiomyoplasty using autologous satellite cells may offer a new treatment. Although the present study does not directly correlate with other findings, we believe that the introduction of an additional unexpected inflammatory response from the Gortex membrane used as a synthetic pericardium in an attempt to reduce adhesion formation may have greatly skewed the results for the ischemia + cells group.

Regional myocardial blood flow analysis of this study indicated that relative blood flow across the groups was not significantly different. Further research is needed to confirm this observation because the population size may not have been large enough to fairly assess significance between groups. There is little experimental information in the literature as to whether transmyocardial laser revascularization improves long-term transmural perfusion and left ventricular function in chronically ischemic myocardium. In a long-term study conducted by Hughes and coworkers, regional contractility and perfusion were found to improve
Horvath and colleagues demonstrated an improved regional contractility at rest in a chronic porcine model of myocardial ischemia, six weeks after transmyocardial laser revascularization treatment (Horvath 1997). However, in contrast, Kohmoto and associates measured regional myocardial blood flow using colored microspheres in an acute ischemic canine model observing no significant differences in regional blood flow, which is consistent with these results (Kohmoto et al 1998). Lutter and colleagues found that while CO2–laser revascularization may have an effect on microperfusion and regional contractility when under stress, compared to their ischemia group of animals three months post chronic myocardial ischemia, they found little to no effect on overall perfusion and global left ventricular function (Lutter and Yoshitake 1998).

In his recent seminar on tissue engineering, Nerem (2005) stated that many of the therapies of the 21st century will be of a combination nature. Our experiment to evaluate the effectiveness of combining two therapies for cardiovascular disease corresponds to Nerem’s prediction, but the results have left us asking the question: What can explain what we found hemodynamically versus what we observed morphologically? Could it be that even though to the naked eye the gross heart sections clearly showed less scar tissue and greater amounts of muscle formation, these morphological improvements do not transfer into physiologic functional improvements? Or could it be that the morphological differences are the first in a series of improvements to be observed? In evaluating cardiac function using the transducing sonometric crystals, we believe that the result of no statistically significant differences for each hemodynamic aspect evaluated can be attributed to a number of factors/limitations.
Limitations

Halothane was used as the inhalation agent in the anesthetic machine to maintain the animals' sedation throughout the surgical procedures. Even though halothane has been used extensively for anesthesia in swine, it has been shown to induce generalized cardiovascular depression and on occasion even elicit bradycardia (Tranquilli et al 1983). These actions are manifested as lowered cardiac output and stroke volume values. The decrease in cardiac output is associated with decreased left ventricular function (Duke et al. 1977). Even though we believe the use of halothane as the anesthetic agent for this experiment could have had negative affects, the fact that halothane was used for all the animals in all surgical procedures ensures equality for comparisons among the groups.

Another limitation that could have influenced our findings was the small population used for this experiment. Miniswine used for research purposes are costly animals to purchase, maintain, and medicate. The sample size is a critical element of an experiment in order to ensure enough study subjects to enable the detection of anticipated effects while determining the minimum sample size required to do so.

Thirdly, the timescale for this experiment could have been too short. For the short duration, the heart will compensate after ischemic injury; for example, a decrease in stroke volume could be compensated by an increase in heart rate to maintain adequate cardiac output to sustain the animal. However, over the long term, compensatory abilities will begin to fail, yielding perhaps a more accurate assessment of the selected therapies.

Also, despite the fact that swine have been shown in investigative literature to be “the animal of choice,” or at least one of them, in experiments such as this where redo open-chest
procedures are required, these animals are challenging specimens (Sanders et al. 1977). The fibrotic adhesions, which were unexpected, were extremely challenging to separate from the pericardium to even be able to proceed with analysis. Additionally, without sedation the miniswine do not easily tolerate typical monitoring methods, such as obtaining blood pressure, heart rate, and temperature values.

**Conclusion**

To date, perhaps the most successful therapy used for cardiovascular disease is a heart transplant. Unfortunately, the demand is increasing while the supply is not. Although our results were not as we would have predicted, from aspects and observable trends in our data as well as findings in other literature, we believe that the fusion of the proven benefits of medical device technology, such as the CO2 heart laser in providing site-specific therapy with the pluripotent attributes of emerging biologic therapeutics, and satellite cell implantation, creates tremendous opportunity for revolutionary change in the way we view and treat disease. Further research, perhaps using larger populations and longer-time intervals, will need to be conducted to discern why such a static difference was observed between morphology and function.
REFERENCES


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                        Poster Presentation at FASEB conference in Washington, D. C.
                        Poster Presentation at FASEB conference in New Orleans, Louisiana


