Fatigue Resistant Muscle by Cell Transplantation and Electrical Conditioning

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Abstract

Congestive heart failure refractory to medical therapy can be managed with cardiac transplantation or artificial heart. Due to the scarcity of donor organs and unsatisfactory long-term outcomes of mechanical devices, skeletal muscle has been used to augment circulatory function. Dynamic cardiomyoplasty, skeletal muscle ventricle, aortomyoplasty, and muscle powered assist devices all require the fatigue resistant muscle to support the failing heart. However, muscle transformation by chronic electric stimulation commonly suffers fibrosis, atrophy, fatty degeneration, and diminished power. Masticatory muscles appear to be highly specialized in mammals and exhibit superior fatigue resistance. Satellite cells isolated from mastication muscles retain their phenotypic characteristics after transplanted into other muscles. Muscle biopsy (~1 g) from canine masseter muscle was used for satellite cell isolation and in vitro proliferation of the myogenic cells. Latissimus dorsi muscles from 12 dogs were randomly assigned into three groups of treatments: 1) control, 2) electrical conditioning, 3) cell transplant + electrical conditioning. After two weeks of vascular delay and eight weeks of conditioning, at the end of fatigue test the power outputs were 0.21±0.02, 0.48±0.07, and 1.30±0.09 watts with the muscle mass of 162±14, 119±18, and 164±15 g for the control, electrical conditioning, and cell transplant + electrical conditioning groups, respectively. Transplantation of autologous satellite cells from jaw closing muscle and chronic electrical stimulation produced skeletal muscle of highly fatigue resistance with preserved mass and power output. This type of muscle could be used for circulatory support or as a permanent power source for assist devices.

Key words: electrical conditioning, muscle fatigue, satellite cell, skeletal muscle.

Basic Appl Myol 13 (2): 83-88, 2003

Congestive heart failure is not a disease per se, but a common clinical manifestation of many cardiovascular disorders that result in extensive myocardial damage and poor ventricular function. It is estimated that 4.79 million Americans suffer from congestive heart failure with 550,000 new causes occur each year [3, 4, 22, 23]. It is the only cardiovascular disorder increasing in prevalence and is the major cause of morbidity and mortality of the aged group. Despite significant advances in medical management [3, 4, 22, 23], the prognosis of congestive heart failure remains poor even when treated with combinations of drugs. Cardiac transplantation has been a proven method for patients with end-stage heart failure. However, the limited donor supply, the required immunosuppression therapy, the accelerated coronary artery disease, and a five year survival rate of 69% [3, 4, 22, 23] render this treatment less than optimal. Mechanical assist devices [16] have been used to support patients with cardiogenic shock, to serve as a bridge for cardiac transplantation, to provide long-term circulatory assistance, and to apply as a bridge for recovery. In addition to high cost and cumbersome power supply, complications of thromboembolism, infection, and mechanical failure produce unsatisfactory clinical results for long-term usage.

Fatigue resistant muscle has been used to augment circulatory function by dynamic cardiomyoplasty, skeletal muscle ventricle, aortomyoplasty, and muscle powered assist devices to support the failing heart. The transformation of skeletal muscle into fatigue resistant muscle by chronic electrical stimulation is one of the major contributions to the success of muscle powered circulatory assistance. However, fibrosis and fatty degeneration plus the diminished mass and power output observed in transformed muscle [5, 7, 12] significantly hinder the optimal outcome. Mastication muscles appear to be highly specialized in mammals and exhibit superior fatigue resistance. These physiological differences may result from fiber-type composition, myosin isoforms, and significant higher mitochondrial content of masseter muscle (Fig. 1). Satellite cells isolated from jaw-closing muscles retain

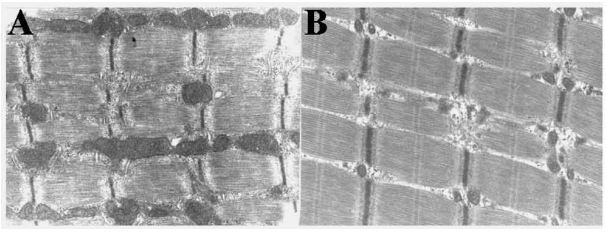

Figure 1. Electron micrographs of masseter muscle (A) and conditioned latissimus dorsi muscle (B). Significantly higher mitochondrial content can be observed in the masseter muscle. Scale bar represent 1 μm.

their phenotypic characteristics after transplanted into other skeletal muscles [9, 10, 17]. This report presents our observations in dogs by transplanting autologous satellite cells from masseter muscle and chronic electrical stimulation to produce latissimus dorsi muscles of highly fatigue resistance with preserved mass and power output.

Materials and Methods

Experimental animal

Mongrel dogs weighing 20 to 25 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 experimental protocol 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. Latissimus dorsi muscles from 12 dogs (left + right = 24 muscles) were randomly assigned into three groups (8 muscles per group) of treatment: 1) control, 2) electrical conditioning, and 3) cell transplant + electrical conditioning.

Isolation, culture, and labeling of satellite cells

Under full anesthesia and sterile surgical conditions, a biopsy sample (~ 1 g) from canine masseter muscle was obtained for cell isolation. The muscle sample was rinsed in 70% ethanol followed by Hank's balanced salts solution without Ca^{++} and Mg^{++} but contained 1% penicillin-streptomycin and 1% amphotericin B. The tissue was minced (~ 1 mm^3) before incubated with 10 ml of enzyme solution in a sterile 15 ml plastic tube. 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% O_2 : 5% CO_2. 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, USA). 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. The cells were cultured with 5 ml of medium in a 25 cm^2 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 sub-cultured every 3 to 4 days to maintain at low density for continual proliferation without differentiation.

The lacZ gene which encodes β-galactosidase in *E. coli* was a commonly used reporter gene for molecular biology studies. The helper-free adeno-associated virus (AAV-LacZ) purchased from Stratagene (La Jolla, CA, USA) was used to label the cultured satellite cells. Since AAV can insert the DNA of interest into the host genome, long-term stable expression was achieved using this method [27]. To prevent the false positive staining resulting from eukaryotic endogenous β-galactosidase activity, the X-gal staining was performed at pH 7.0 for 4 hours [1]. When AAV-LacZ was used to label the cultured satellite cells, long-term high efficiency and high specificity of labeling was achieved (Fig. 2) under *in vitro* condition without interfering the myogenic capability of the labeled satellite cells [13].

Mobilization, cell implantation, and electric conditioning of Latissimus Dorsi muscle

Under sterile surgical condition latissimus dorsi muscles were freed from their insertions, chest wall, and subcutaneous tissue but with careful preservation of neurovascular bundle and humeral insertion. Each muscle was divided into 34 to 38 areas of 1.5 x 1.5 cm for the upper 1/3 of the muscle and 2 x 2 cm for the remaining. At the center of each area, 0.1 ml of cell suspension (~ 1 million cells in serum free culture medium) was injected at 3 mm depth for the upper 1/3 of the muscle and at 1.5 mm depth for the rest of the muscle. The depth of injection was controlled by a plastic collar over the hypodermic needle. The control and electrical conditioning muscles received serum free culture medium in the same manner. To prevent adhesion and collateral revascularization, the Gore-Tex surgical membrane (WL Gore & Assoc. Inc.,

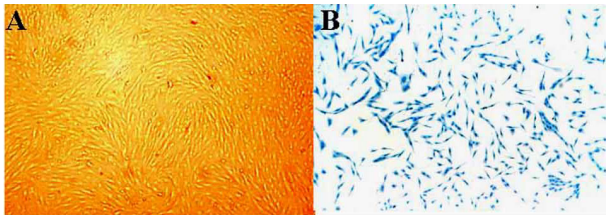

Figure 2. Cultured canine satellite cells (A) from masseter muscle, after transfected with AAV-LacZ and reacted with X-gal, almost all cells (B) expressed β-galactosidase activity. Original magnification is 40X.

Flagstaff, AZ, USA) was used to encase the latissimus dorsi muscle. The muscle was fixed to its original anatomical location before the wound closed in layers.

A paraneural bipolar electrode (Medtronic Inc., Minneapolis, MN, USA) covering 180° of the thoracodorsal nerve circumference was positioned. The electrode was attached to the nerve near its junction at the latissimus dorsi muscle with the superior conductor being the cathode. If both left and right latissimus dorsi muscles were stimulated, the custom-made Y lead (YY0050931R, Medtronic Inc., Minneapolis, MN, USA) was used to allow identical pacing of both muscles with one pulse generator. Using a cuff electrode the threshold voltage (minimum voltage to produce measurable tension) is below 0.5V and plateau voltage (minimum voltage for maximum force generation) is about 1.0V [18]. To ensure full stimulation without nerve injury, supramaximal voltage (2.5V) was used during muscle conditioning. After two weeks of vascular delay, the Medtronic Itrel neuromuscular stimulator (Medtronic Inc., Minneapolis, MN, USA) was used to condition the muscle simulating clinical stimulation protocol [6]. The stimulator was set with 180 ms on and 820 ms off cycles with 210 μs pulse width at 10 Hz (weeks 3 and 4), 15 Hz (weeks 5 and 6), 20 Hz (weeks 7 and 8), and 30 Hz (weeks 9 and 10) to give 60 stimulations per minute at 1, 2, 3, and 5 pulses per stimulation. At the end of 10 weeks, each muscle was subjected to fatigue test.

Quantification of skeletal muscle work capacity

The power output and fatigue resistance of each muscle were measured by our published procedure [24, 25]. Precise quantification of power output by the muscle-actuated pump coupled to the mock circulatory system was applied (Fig. 3). The ease to perform and precision in quantification were the major advantages of this procedure.

Total Power output = Kinetic Energy + Potential Energy

Kinetic Energy (Joule) = Pressure Development x Area x Distance
= Pressure Development x Volume Displacement

Potential Energy (Joule) = ½ mv^2
= ½ (Mass of Water) (Mean Velocity)^2

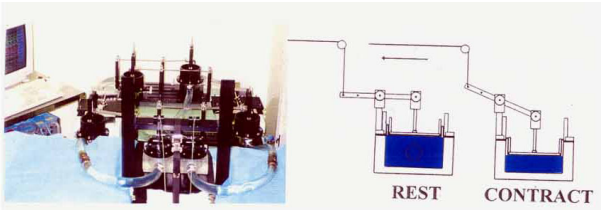

Figure 3. The muscle-actuated pumps coupled with mock circulatory system for quantification of power output. The muscle-actuated pump is made of Plexiglas pumping chamber with a piston transmitting the force into hydraulic power with a rolling diaphragm. Energy transmission losses from the muscle to hydraulic fluid are negligible due to the frictionless nature of the rolling diaphragm.

The pressure was measured in mmHg that was converted to newtons (1 mmHg = 0.01333 N/cm^2). The volume displacement was equal to the piston head area (56 cm^2) times the distance the piston moved. The volume displacement measured by an in-line flow probe could also be used. Kinetic energy = pressure development (mmHg) x volume displacement (ml) x 1.333 x 10^{-4}. The mass of water = volume displacement (ml) x 1 (density of water) = g. The mean velocity = volume displacement/(ejection time) (cross section area). By converting to the proper units (Joule), the total energy was obtained. Since 1 watt = 1 joule/s, the work performed by the muscle could also be easily derived.

Histological and electron microscopic evaluations

At the end of the fatigue study, the muscle was isolated, the surgical membrane removed, and trimmed for determining the total mass. Representative muscle samples from the proximal, middle, and distal portions of lateral, oblique, and transverse segments were obtained [21] for immunohistological, histochemical, and morphological evaluations. After the tissue samples were prepared for paraffin or frozen sections, the myosin ATPase activity was studied with preincubation in acid (pH 4.3) or alkaline (pH 10.4) solution. The X-gal staining was performed at pH 7.0 for 4 hours at 37°C without light for all the samples as the published method [1]. Antibodies against specific myosin isoforms (obtained from Dr. Hoh) were also used to determine the muscle fibers expressing superfast myosin [9, 10, 17]. The procedure modified from the published method [15, 21], routinely used in this laboratory was applied to the samples. Tissue samples were stained for Hematoxylin and Eosin, Masson's Trichrome, and Oil Red O [15], for histological evaluations. For electron microscopy, the tissue samples were fixed in 0.1 M cacodylate buffer containing 2% glutaraldehyde before post fixed in osmium tetroxide, dehydrated, embedded, and sectioned. The sections were mounted and stained before being viewed with the electron microscope as in our earlier publication [14].

Results

Muscle mass and gross morphology

At the end of the fatigue test, each muscle was isolated and the surgical membrane removed (Fig. 4) before determining the muscle mass. The average weight of latissimus dorsi muscles for control group was 162 ± 14 g, for electrical conditioned group was 119 ± 18 g, and for the cell implant + electrical conditioned group was 164 ± 15 g. The muscle mass for electrical conditioned group was significantly lower ($p < 0.05$) than the other groups. A thoracodorsal nerve sample was obtained from each muscle at a site just below the paraneural electrode and subjected to histological evaluation. The motor nerve from control and electrical conditioned muscle showed similar morphology (Fig. 4). These results indicating the paraneural electrode and muscle conditioning program did not cause any observable damage to the nerve system.

Power output of Latissimus Dorsi muscle

Quantification of power output for each muscle was accomplished by using the muscle-actuated pump coupled to the mock circulatory system. The initial power outputs of the cell implant + electrical conditioned muscles were about the same as the control muscles (1.6 watts) while the conditioned muscles had only 75% power outputs as compared to these groups. At the end of two hours of fatigue test, the control muscles lost 80% of their power outputs while the cell implant + conditioned muscles retained better than 80% of their initial power outputs. Although 40% of initial power outputs were maintained by the conditioned muscles, their initial power outputs were significantly lower than all other muscles (Fig. 5). At the end of the fatigue test, the power outputs were 0.21 ± 0.02 watts for control group, 0.48 ± 0.07 watts for electrical conditioned group, and 1.30 ± 0.09 watts for cell implant + electrical conditioned group. Satellite cell transplantation and chronic electric stimulation produced latissimus dorsi muscles of highly fatigue resistance with preserved mass and power outputs.

Histological observations

Latissimus dorsi muscle samples were obtained before cell implantation, at 2, 5, and 10 weeks after transplantation of autologous satellite cells isolated from masseter muscle and labeled with LacZ. The transplanted cells survived in the muscle and either fused with existing muscle fibers or formed new muscle fibers before gradually transforming the entire muscle (Fig. 6). The changes in myosin ATPase activity and expression of superfast myosin followed the similar pattern. Satellite cell transplantation either prevented or repaired the muscle injury as evidenced by the histological observation (Fig. 7) of conditioned muscles vs. cell implant + conditioned muscles. The infiltration of inflammatory cells, vacuolization and degeneration of muscle fibers, as well as accumulation of collagen fibers were quite obvious for the muscles without cell implantation. When the muscle samples were stained with oil red O (Fig. 7), the increase of interfascicular fat content without fatty degeneration, vacuolization, and fibrosis were noticed for the cell implant + conditioned muscles as compared to the conditioned muscles.

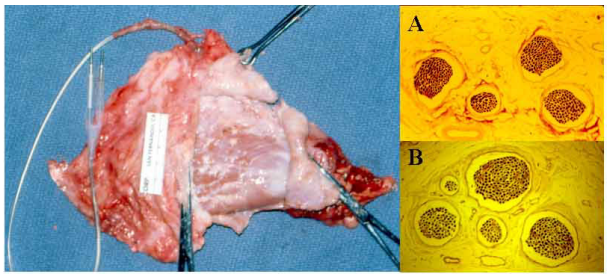

Figure 4. Isolated latissimus dorsi muscle encased in surgical membrane with part of the membrane pulled away. The thoracodorsal nerve next to stimulating electrode showing normal morphology for control (A) and stimulated muscle (B).

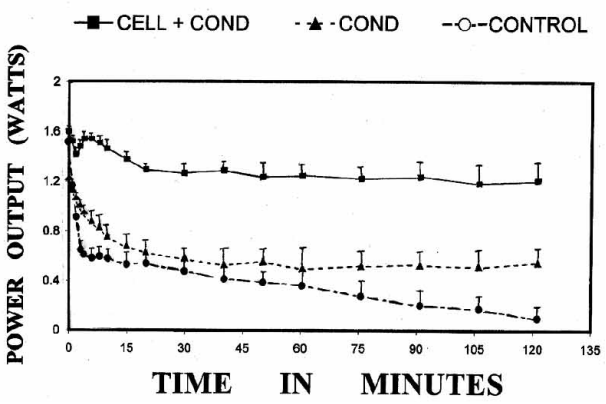

Figure 5. Power outputs of latissimus dorsi muscles. The control and cell implant + conditioned (CELL + COND) groups have similar initial power outputs. The conditioned muscles (COND) lost about 25% of their initial power outputs. CELL + COND group maintained outstanding power output till the end of fatigue test (120 min).

Figure 6. Latissimus dorsi muscle samples with LacZ labeled satellite cell implantation after reacted with X-gal. A = before cell implantation; B = at 2 weeks, C = at 5 weeks, and D = at 10 weeks after cell transplantation. The cells fused with existing myofibers or formed new fibers expressing β-galactosidase activity. Scale bar equals to 1 cm.

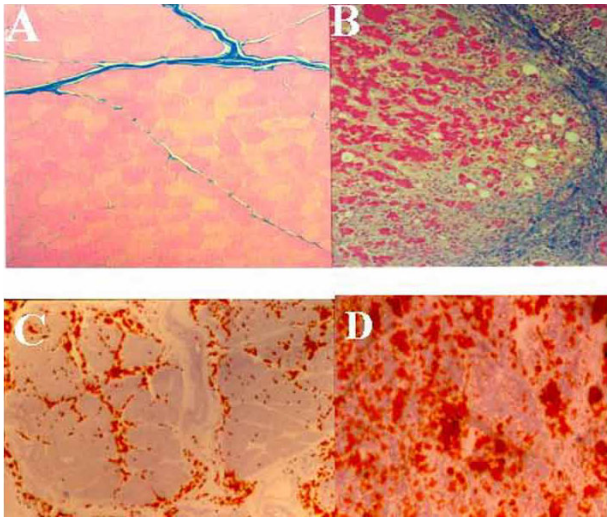

Figure 7. Micrographs of conditioned latissimus dorsi muscle samples with (Left) or without (Right) cell implantation. Masson's Trichrome staining for upper panel and Oil Red O staining for lower panel. Satellite cell transplantation prevented fibrosis and degeneration of the conditioned latissimus dorsi muscle (A & C). Original magnification is 20X.

Discussion

It is not new to utilize living tissue with growth potential to repair cardiac abnormalities. Various muscles have been transplanted into, onto, or around the heart over the past decades in an effort to enlarge the cardiac chamber, to reinforce myocardial defects, or to augment cardiac output. However, skeletal muscle fatigue and associated atrophy, fibrosis, and degeneration have been the major limitations for the success of these procedures. The skeletal muscle can be injured due to the surgical trauma and ischemia (termination of collateral blood supply), limited capacity of oxidative metabolism, over stimulation, insufficient relaxation time, and inadequate blood supply during functional support. The ischemic injury can be relieved by vascular delay (allow time for revascularization) and insufficient relaxation time can be overcome by demand stimulation [19, 20]. This paper presented an innovative approach to increase oxidative metabolic capacity, fatigue resistance, and power output by transplanting autologous satellite cells from masseter muscle into latissimus dorsi muscles.

Since the migration of satellite cells has been clearly documented [11, 26], the myogenic cells from masseter muscle should cover a significant portion of latissimus dorsi muscle. The functional recovery of irreversibly injured muscles have been observed after satellite cell implant [2]. Based on our observation and the published report [2, 11, 26], the transformation of canine latissimus dorsi to a powerful and fatigue resistant muscle with the characteristics of masseter muscle is the expected outcome. Our previous study, has proved that nerve activation is the primary mechanism of muscle stimulation [18]. The implanted satellite cells will proliferate and differentiate to form new myotubes or fuse with existing myofibers. The newly formed myotubes will be innervated by the motor nerve of the latissimus dorsi muscle while the existing myofibers already have their innervation [8, 9]. This makes the nerve stimulation of the entire latissimus dorsi muscle possible even with new myofibers formed from implanted cells.

Myogenic cells forming jaw-closing muscles are from cephalic mesoderm which is distinct from somatic mesoderm that forms limb and trunk muscles. Masticatory muscles appear to be highly specialized in mammals and exhibit different contractile properties. These physiological differences probably result from fiber-type composition and myosin isoforms. The cross-transplantation studies (transplanting a piece of jaw muscle into a limb muscle bed and allow it to regenerate and reinnervate) show that the transplanted mastication muscle retains its specific myosin isoforms. Before transplantation, the jaw muscle fibers are killed by chemical treatment leaving the satellite cells to form new muscle. Therefore, the satellite cells from mastication muscles retain their phenotypic characteristics independent of environmental and neuro regulations. This is further supported by the aneural regeneration of jaw muscle which also express the specific myosin [9]. In addition to specific myosin isoforms, the significantly higher mitochondrial content of masseter muscle (Fig. 1) may contribute to the superior fatigue resistance of jaw-closing muscles. Transplantation of autologous satellite cells from masseter muscle and chronic electrical stimulation produce skeletal muscle of highly fatigue resistance with preserved mass and power output. This type of muscle can be used for circulatory support or as a permanent power source for assist devices.

Acknowledgements

Our studies have been supported by VA Merit Review Grant; NIH grant HL54286, HL72138; and American Heart Association grant 0051458B, 0255009B.

Gore-Tex Surgical Membranes were generously provided from WL Gore & Assoc. Inc.

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