A NEW SYSTEM AND PARADIGM FOR SIMULTANEOUS CHRONIC STIMULATION OF DENERVATED MUSCLE IN MULTIPLE ANIMALS

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ABSTRACT

Traditionally, animal studies employing electrical muscle stimulation for conditioning denervated muscle rely on implantable stimulators and 24 hour based stimulation paradigms. While these stimulators provide the necessary current to cause muscular contraction, they have problems with battery life, programmability, and long term robustness. Continuous 24 hour stimulation, while shown to be effective in animals, is not easily translatable to a clinical setting. It is also difficult to evaluate animal comfort and muscular contraction throughout a 24 hour period. We have developed a system and stimulation paradigm that can stimulate up to five animals at one time for one hour per day. The constant current stimulator is a USB powered device that can, under computer control, output trains of pulses with selectable shapes, widths, durations and repetition rates. It is an external device with no implantable parts in the animal except for the stimulating electrodes. We have tested the system on two groups of rats with denervated gastrocnemius muscles. One group was stimulated using a one hour per day stimulation paradigm for one month, while the other group had electrodes implanted but received no stimulation. Muscle weight and twitch force were significantly larger in the stimulated group than the non-stimulated group. Presently, we are using the stimulator to investigate electrical stimulation coupled with other therapeutic interventions that can minimize functional deficit after peripheral nerve injuries.

INTRODUCTION

Peripheral nerves are the anatomical structures that connect our central nervous system to the structures that move our joints - muscles. When peripheral nerves are cut or crushed during injury, the muscles lose their connection with the central nervous system and become denervated. In order for full functional recovery to occur, peripheral nerves must grow from the site of injury until they reach the muscle and form functional connections with the muscle. Clinically, functional recovery after peripheral nerve injuries is poor[1]. Because peripheral nerves regenerate at a rate of 1mm per day, injuries that are more proximal have much longer periods of muscle denervation. Progressive muscle atrophy usually follows long term denervation with a loss of muscle mass, force, motor function and an increase in collagenization and fibrosis of the tissue[2,3,14].

Electrical stimulation of denervated muscle to prevent atrophy has been studied as early as the 1930's[4]. However, the use of stimulation as a clinical therapy is not widely accepted due to a lack of standards and questions of efficacy[5]. Indeed, efficacy is the main concern since different studies show both positive and negative effects of stimulation[6]. These negative effects may be due to a lack of stimulation intensity needed to reach deep muscle fibers when using surface electrodes, incorrect frequency selection for stimulation, or stimulation protocols with long periods of rest between stimuli[7,8]. Nevertheless, intense stimulation of denervated muscle has been shown to have beneficial effects in human subjects and thus is a worthwhile approach to maintaining muscle mass and force[9]. Numerous animal studies have been conducted using implantable stimulators to provide electrical muscle stimulation to denervated muscle[10,11,12,16]. The bulk of these utilized stimulation paradigms that consisted of 24 hours of continuous stimulation, something not easily translatable to a clinical setting unless expensive implantable stimulators are used[13]. It is also difficult to assess the level of muscular contraction over a 24 hour period.

In this paper, we present a 1 hour per day stimulation paradigm that can be easily translatable to a clinical setting, along with an external system designed to stimulate five animals simultaneously using this paradigm.

METHODS

System Design

A block diagram of our system is shown in Figure 1. The stimulator utilizes a universal serial bus (USB) to provide power and communicate with the host computer. Stimulation parameters (pulse width, frequency, amplitude, polarity) are sent via the host computer as text commands and interpreted by the

stimulator's microcontroller acting as a serial port emulator (PIC18F4550). The microcontroller uses the timing parameters to switch an isolated h-bridge. The h-bridge is coupled to a voltage controlled constant current source used to provide biphasic stimulus pulses. The output of the h-bridge can be directly connected to an animal as a single channel device, or connected to a five channel break out box. The break out box is a set of analog switches (MAX 4623) controlled by the microcontroller. This allows each of the five channels to have individual stimulus settings. Stimulus voltage is provided by a 5-15V step up converter (MAX 630) that is isolated on the input side using a 5V isolated DC/DC converter (DCH010505S). A maximum of 15V has been used in previous studies and shown to provide sufficient stimulus amplitude to contract denervated muscle[10].

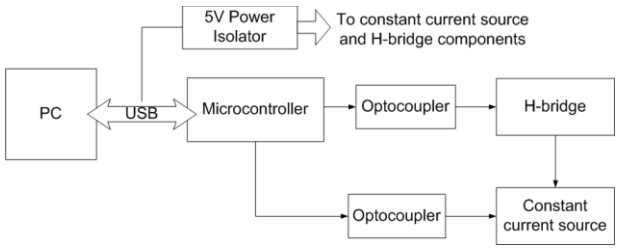

Figure 1 – Block diagram of stimulator

Animals

Ten male Lewis rats (Charles River, Quebec, Canada) weighing 250-350g were used for this study. This strain was chosen as it shows the least self-mutilation following surgery. All housing, surgical procedures, analgesia and assessments were performed according to the Canadian Council on Animal Care Guidelines, using protocols approved by the Animal Care Committee at McMaster University.

Surgical Procedure

The animals were randomly assigned to either a stimulated group or an unstimulated group. Each group had the right gastrocnemius muscle denervated as described previously[14]. Teflon coated, stainless steel stimulating electrodes (Cooner Wire, AS 631), with ends bared of insulation, were implanted into the belly of the denervated muscles of both groups using an electrode suture complex[15] to minimize electrode migration. Slack wire was then coiled near the biceps femoris to allow for limb movement and animal growth. The electrode wires were threaded subcutaneously beneath the dorsal trunk skin, sutured in place, and externalized at the nape. The ends of the wire were bared for connection to the stimulator.

Stimulation Paradigm

For the stimulation paradigm to be easily translatable to a clinical setting, we chose a 1 hour duration for muscle stimulation. Previous studies utilizing 1 hour stimulations have shown some benefit[16]. However, those studies utilized a moderately low frequency (20Hz) to stimulate a primarily fast twitch muscle (tibialis anterior) and elicited unfused tetanic contractions[16]. We opted to use a frequency more suited towards a fast twitch muscle. A frequency of 100 Hz has been used in previous studies[10] and elicited fused tetanic contractions in the gastrocnemius. A biphasic tetanic train of 400 ms duration (40 pulses at 100 Hz) was used with a pulse width of 200 µs per phase. We aimed to match previous studies and therefore elicited 1200 contractions per stimulus session (one contraction every 3 seconds). However, this fatigued the muscle significantly and so we moved to our current protocol of 600 contractions per day (one contraction every 6 seconds). The stimulus amplitude was adjusted for each animal until a visually strong contraction was produced. Stimulation began two days post-operatively, and each of the animals in the stimulated group underwent daily stimulation for 4 weeks (weekdays only).

Muscle Assessment

After one month, the animals were anesthetized using Halothane (2%) and the gastrocnemius muscle in both hind limbs exposed, dissected free of the soleus and plantaris muscles, and connected to a force transducer (Grass FD03). Two fine needle electrodes were placed in the belly of the muscle and served as stimulating electrodes. Muscle length was adjusted to the optimum length for force generation and maximum twitch forces, time to peak, and half-relaxation times were measured in both limbs with the left hind limb serving as a fully innervated control. A fatigue test was then initiated which consisted of 13 pulses at 40 Hz delivered once every second[17]. The fatigue index was calculated by measuring the maximum tension at 2 minutes and dividing it by the maximum tension generated by the first stimulus. Once the force and fatigue tests were completed, the muscle was excised, weighed, and immediately immersed in liquid nitrogen-cooled isopentane. 8-µm transverse sections were then taken from the frozen muscle and stained using hematoxylin and eosin for viewing under a light microscope. Ten images of each muscle were taken spanning the entire cross-section using a Nikon D300 camera adapted to a Carl Zeiss Universal light microscope and muscle fiber area was measured using ImageJ software (NIH).

Statistical Analysis

Mean values are presented along with the standard error of the mean. An unpaired T test was used to compare the denervated-stimulated group to the purely denervated group. In cases where the stimulated, unstimulated, and control groups (fully innervated) are compared, a one way ANOVA was used with a Bonferroni post hoc test. Significance was defined as $p < 0.05$.

RESULTS

One of the animals in the denervated-stimulated group was not assessed physiologically, as the muscle was damaged during the dissection and could not be stimulated.

To remove any variability due to animal weights, muscle weight and twitch force were expressed as percentages of the control (fully innervated contralateral limb). For both weight and force, the ratios were significantly larger in the stimulated group than the unstimulated group (Table 1). The fatigue test showed that the stimulated group had a significantly higher fatigue index (more resistant to fatigue) than the fully innervated controls (Table 2). There was no significant difference between denervated and control groups.

The stimulated group also had significantly longer contraction and half-relaxation times compared to the denervated and control groups.

Table 1: Muscle weight and maximum twitch force

	Denervated	Denervated-Stimulated
Muscle Weight (% control)	32.8 ± 0.37 n = 5	43.0 ± 2.55* n = 5
Twitch Force (% control)	20.2 ± 1.50 n = 5	35.0 ± 2.94* n = 4

*Numbers represent mean values ± SEM. *Denotes significant differences between groups, p < 0.05.*

Table 2: Fatigue index (FI), time to peak (T_{peak}), and half-relaxation time ($T_{1/2R}$)

	Control	Denervated	Denervated-Stimulated
FI	0.11 ± 0.03 n = 10	0.23 ± 0.02 n = 5	0.28 ± 0.05* n = 4
T_{peak} (ms)	39.8 ± 1.46 n = 10	39.2 ± 2.59 n = 5	56.8 ± 6.24* n = 4
$T_{1/2R}$ (ms)	25.9 ± 2.07 n = 10	27.88 ± 1.29 n = 5	48.95 ± 6.91* n = 4

*Numbers represent mean values ± SEM. *Denotes significant difference compared to the control group, p < 0.05*

The overall structure of the muscle fibers was better maintained in the denervated-stimulated group than the unstimulated group. There was clearly less connective tissue in the stimulated group and the fibers appeared more tightly packed, resembling those of fully innervated muscles rather than denervated muscles. Fiber area was significantly larger in the stimulated group compared to the unstimulated group (Figure 2). However, both were less than the control (Table 3).

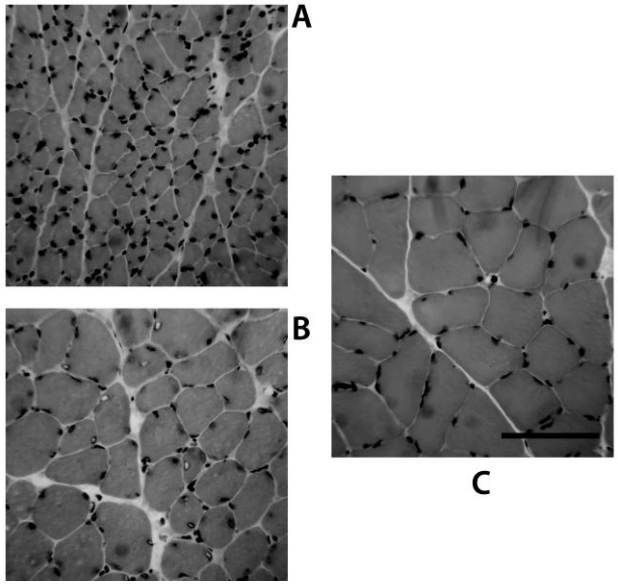

Figure 2 – Histological sections of the medial gastrocnemius muscle stained with hematoxylin and eosin (H&E). (A) denervated muscle; (B) denervated and stimulated muscle; (C) fully innervated muscle. Bar represents 100µm.

Table 3: Muscle fiber area

	Control	Denervated	Denervated-Stimulated
Area (μm^2)	2605 ± 45.7† n = 489	618.7 ± 7.39 n = 1297	949.6 ± 12.09* n = 1508

*Numbers represent mean values ± SEM. *Denotes significant difference compared to the denervated group, p < 0.05. †Denotes significant differences compared to denervated and stimulated groups, p < 0.05.*

DISCUSSION

The data in this pilot study verify that our stimulus paradigm is effective at increasing muscle weight, twitch force, and fiber area. During tetanic stimulation, the control force values we recorded were out of the transducer's range and were not included in the results of Table 1.

Denervated primarily fast twitch muscle like the gastrocnemius normally undergoes fiber type

conversion from fast glycolytic to fast oxidative types (IIb to IIa)[18]. This would explain the increase in fatigue resistance in both the denervated and denervated-stimulated groups compared to innervated controls. However, this does not explain the increase in contractile and half-relaxation times. Although a stimulation frequency similar to the firing frequency of fast twitch fibers was chosen (100 Hz) to avoid fiber conversion, the results show that the contraction and half-relaxation times are more akin to those of slow twitch fibers. One reason for this could be that chronic denervation increases the membrane time constant or the refractive period[19] resulting in a lower effective stimulation rate than the applied 100 Hz. ATPase fiber typing histology will be done in the future to explore whether this paradigm converts fast fibers into slow fibers.

Traditionally, denervated muscle studies conducted on animals utilized implantable stimulators that are costly and require special technical expertise and manufacturing facilities. Our system was built using a majority of standard components that are easily obtainable at low cost, making the stimulator an economical solution for animal studies requiring stimulation. The stimulator could easily produce the necessary amplitudes to elicit strong contractions in denervated muscle. Though the animals were fully awake during the stimulation period, they were restrained in custom designed restrainers that minimized movement. The animals showed minimal discomfort throughout the stimulation period. Further, designing the system to reliably stimulate up to five animals concurrently considerably increased laboratory efficiency.

Finally, stimulated muscle force and weight did not approach control values, supporting the hypothesis that contractile input alone is not adequate to fully maintain denervated muscle. Our new electrical stimulation system and paradigm will be useful for investigating the combination of electrical stimulation and other therapeutic interventions to improve functional recovery after nerve injury.

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