

A New System and Paradigm for Chronic Stimulation of Denervated Rat Muscle

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Abstract

Traditionally, animal studies employing electrical stimulation for conditioning denervated muscle rely on 24-hour-based stimulation paradigms, most employing implantable stimulators. 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 tested the system on two groups of rats with denervated gastrocnemius muscles. One group was stimulated using a one-hour-per-day, 5-days-per-week 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 deficits after peripheral nerve injuries.

Keywords: Electrical muscle stimulator, Peripheral nerve injury, Denervated muscle, Chronic electrostimulation, Electrophysiology, Muscle atrophy

1. 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, particularly if there is a prolonged delay before nerve-muscle contact is re-established [1]. Because peripheral nerves regenerate at a rate of 1 mm per day, injuries that are more proximal produce

much longer periods of muscle denervation. Progressive muscle atrophy usually follows long-term denervation with a loss of muscle mass, muscle spindles, force and motor function and an increase in collagenization and fibrosis of the tissue [2-4].

Electrical stimulation of denervated muscle to prevent atrophy was studied as early as the 1930's [5]. However, the use of stimulation as a clinical therapy is not widely accepted due to a lack of standards and questions of efficacy [6]. Indeed, efficacy is the main concern, since different studies show both positive and negative effects of stimulation [7,8]. 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 [9,10]. 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 [11]. Numerous animal studies have been conducted

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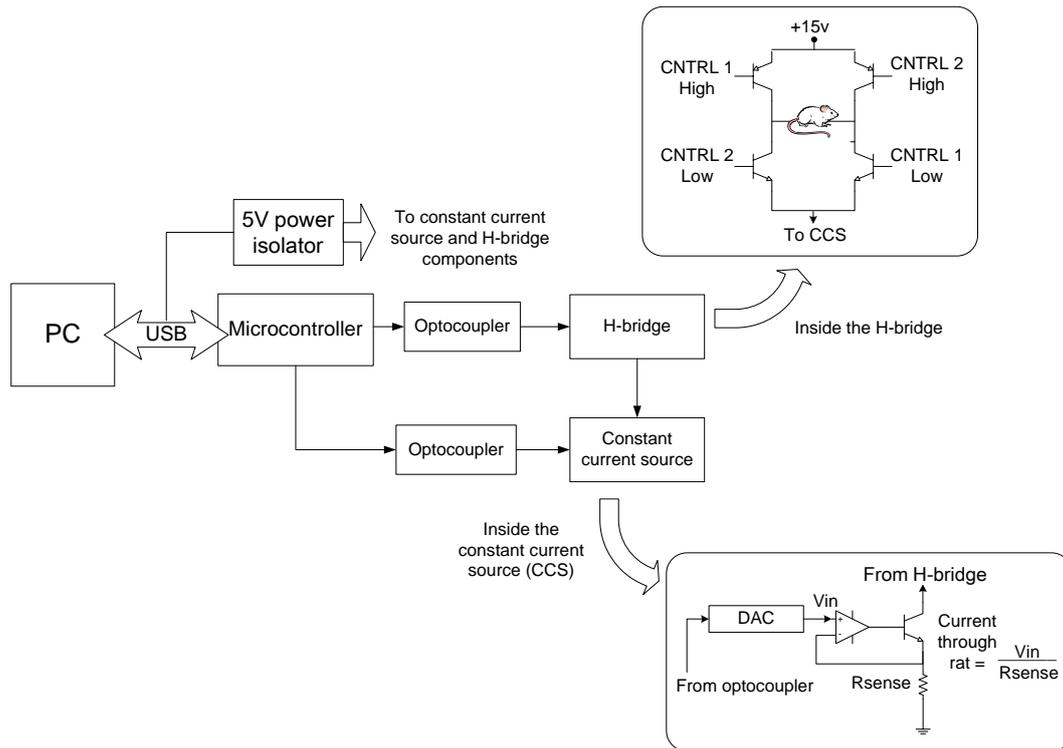


Figure 1. Block diagram of the stimulator. The expanded views detail the components of the H-bridge and constant current source.

using implantable stimulators to provide electrical muscle stimulation to denervated muscle [12-15]. These stimulators are costly, especially when conducting large-scale studies involving numerous animals. They also require special manufacturing facilities since their small size requires custom-designed integrated circuits and exclusively surface-mounted technology. The power output is always limited, and batteries may need to be changed during long-duration studies. The bulk of these studies also used stimulation paradigms that consisted of 24 hours per day of intermittent stimulation, something not easily translatable to a clinical setting unless expensive FDA-approved implantable stimulators are used [16]. It is also difficult to assess the level of muscular contraction over a 24-hour period.

In this paper, we present an external system designed to stimulate up to five animals simultaneously using a 1-hour-per-day, 5-days-per-week stimulation paradigm. The choice of an external system over an internal one was largely that of cost, ease of assembly, robustness and eventual clinical applicability. Moreover, a 1-hour-per-day/5-days-per-week stimulation paradigm is easily translatable to a clinical setting.

2. Materials and methods

2.1 System design

A block diagram of our system is shown in Fig. 1. The stimulator utilizes a universal serial bus (USB) to provide power and communication with the host computer. Stimulation parameters (pulse width, frequency, amplitude, polarity) are sent as text commands via custom LabVIEW (National Instruments) software running on the host computer and

interpreted by the stimulator's microcontroller acting as a serial port emulator (PIC18F4550, Microchip Technology Inc.). This microcontroller was selected due to its easy USB interfacing. It 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. This current source consists of a transistor driven by an operational amplifier with a stimulation amplitude reference voltage provided by a digital to analog converter (MCP4821, Microchip Technology, Inc.).

For chronic electrical muscle stimulation, biphasic pulses are necessary to prevent net ion flow that could result in tissue damage or electrode corrosion [17]. The purpose of having an isolated stimulator output is to ensure that no common ground loops are present and that the stimulus cannot be shunted through the animal's body to a common ground sink. As well, isolation is required to allow simultaneous recording of electrophysiological signals. Isolation is implemented in two ways: control signals arriving from the microcontroller are digitally isolated using optocouplers (4N33, Fairchild Semiconductor); stimulus voltage is provided by a 5-15 V step-up converter (MAX 630, Maxim Integrated Products, Inc.) that is isolated on the input side using a 5 V isolated DC/DC converter (DCH010505S, Texas Instruments). The designed 15-V maximum stimulator output has been shown in other studies to provide sufficient stimulus amplitude to contract denervated muscle [12]. The output of the H-bridge can be directly connected to an animal as a single-channel device, or connected to a five-channel breakout box (not shown in Fig. 1) for stimulation of multiple animals. The breakout box is a set of analog switches (MAX 4623, Maxim Integrated Products, Inc.) controlled by the microcontroller. This allows each of the five

channels to have individual stimulus settings. System testing, using a resistance of 620 Ω placed across the stimulator outputs, showed it met design specifications and produced mono or biphasic pulses at selected amplitudes, durations, and repetition frequencies. This resistance is similar to that of denervated and stimulated muscle [12].

2.2 Animals

Ten male Lewis rats (Charles River, Quebec, Canada) weighing 250-350 g were used for this study. This strain was chosen as it shows the least self-mutilation following surgery [18]. 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.

2.3 Surgical procedure

The animals were randomly assigned to either a stimulated or non-stimulated group. Each group had the right gastrocnemius muscle denervated, as described previously, by cutting the tibial nerve approximately 13 mm from its entry point into the gastrocnemius muscle. The free distal stump was sutured to minimize extraneous innervation from other axons, and the proximal stump of the nerve was sutured onto the biceps femoris muscle to avoid reinnervation from proximal tibial nerve axons [4]. Teflon coated, stainless steel (Cooner Wire, AS 631) stimulating electrodes with ends bared of insulation were implanted into the belly of the denervated muscles of both groups using an electrode suture complex [19] 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. This approach worked very well in chronic stimulation and caused no animal discomfort or infections at the wire exit sites.

2.4 Stimulation paradigm

In order for the stimulation paradigm to be easily translatable to a clinical setting, we chose to use a 1-hour/day, 5-days/week muscle stimulation protocol. Previous studies using 1-hour durations for stimulation had shown some benefit. However, those studies employed a relatively low pulse repetition frequency (20 Hz) to stimulate a primarily fast twitch muscle (rabbit tibialis anterior) and elicited brief, unfused, tetanic contractions [15]. We opted to use a frequency of 100 Hz, more suited to a fast twitch muscle, which had also been used in previous studies [8,12] to elicit fused, tetanic contractions of the rat gastrocnemius muscle. A biphasic train of 400 ms duration (40 pulses at 100 Hz) was used with a pulse width of 200 μ s per phase. In a preliminary study, we attempted to effectively match previous protocols and elicited 1200 contractions per stimulus session (one contraction every 3 seconds, similar to [15]). However, this fatigued the muscle significantly, and we consequently moved to our current protocol of 600 contractions per hour (one contraction every

6 seconds). The stimulus amplitude was adjusted for each animal until a visually strong contraction was produced.

2.5 Muscle assessment

After the one-month experimental period, the animals were deeply anesthetized using halothane (5% induction, 2% maintenance) and the gastrocnemius muscle in both hind limbs was 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 (T_{peak}) and half-relaxation times ($T_{1/2R}$) were measured in both limbs, with the left hind limb serving as the fully innervated control for each denervated muscle. A fatigue test was then initiated which consisted of 13 pulses at 40 Hz delivered once every second [20], which was different from our stimulus protocol but used by others for endpoint fatigue testing [15,21,22]. The fatigue index (FI) was calculated by measuring the maximum tension after 2 minutes and dividing it by the maximum tension generated by the first stimulus. Once the force and fatigue tests were completed, the animal was sacrificed using an overdose of halothane and cervical dislocation. The muscle was excised, weighed, and immediately immersed in liquid nitrogen-cooled isopentane. Using a cryostat, 8- μ m 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 light microscope, and muscle fiber cross-sectional area was measured using ImageJ software (NIH).

2.6 Statistical analysis

To remove any variability due to animal weights, muscle weight and twitch force were expressed as percentages of the control (fully innervated, contralateral) limb. An unpaired *t*-test was used to compare the stimulated group to the unstimulated group. In cases where the stimulated, unstimulated, and unoperated groups were compared, a one way ANOVA was used followed by a Bonferonni *post hoc* test. Significance was defined as $p < 0.05$.

3. Results

Stimulations began two days post-operatively, and each of the animals in the stimulated group underwent daily stimulation for 4 weeks (weekdays only). A biphasic train of 400 ms duration (40 pulses at 100 Hz) with a pulse width of 200 μ s per phase was given every six seconds for one hour. Though the animals were fully awake during the stimulation period, they were restrained in custom-designed restrainers that minimized movement. The restrainer with a rat inside is shown in Fig. 2. The animals showed minimal discomfort throughout the stimulation period. One of the five animals in the stimulated group was not assessed physiologically, as the muscle was damaged during the dissection and could not be stimulated.



Figure 2. Rat in custom-designed restraining device. Right leg is exposed for easy visualization of contractions.

Maximum muscle twitches were elicited to provide a measure of the loss in the denervated muscle's force output. Figure 3 shows the relative losses in muscle weight and maximum twitch forces for both denervated groups while Fig. 4 shows typical twitches obtained from one animal per group. These figures show that there was significantly less force loss in the stimulated than in the unstimulated group.

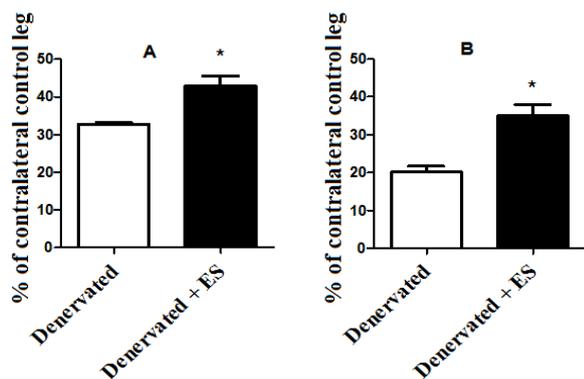


Figure 3. (A) Muscle weight and (B) twitch force ratios in rat gastrocnemius muscle 1 month after denervation or denervation and electrical stimulation (ES). Values represent the ratio of the denervated muscle to its contralateral unoperated control leg and are expressed as mean \pm standard error. The means of the stimulated group in both the weight and twitch force were significantly higher than those of the unstimulated group ($*p < 0.05$). $n = 5$ in the denervated group and $n = 4$ in the stimulated group.

The shape and duration of the twitch are indicative of the fiber type, whether fast or slow twitch. While the times to peak force and half relaxation force in Table 1 were the same for control and unstimulated groups, showing that there was no change in contractile properties of the muscle fibers during one month of denervation, they were significantly increased for the stimulated group. Thus, when the muscle is electrically stimulated, there is a change in some muscle fibers from fast to slow twitch. Fatigue tests were done to examine if there were changes in the muscle's resistance to fatigue during one month of denervation, which would also indicate a change in fiber structure and/or metabolism. Table 1 shows that the stimulated group had a significantly higher FI than the fully innervated controls. Although the mean FI for the denervated group also

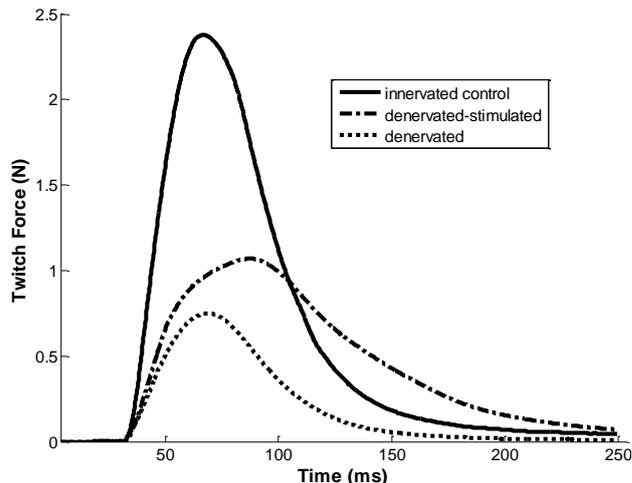


Figure 4. Force profiles of the twitch response for fully innervated muscle, denervated muscle, and denervated stimulated muscle. The profile of the stimulated muscle shows a lengthening of the contraction and half-relaxation times, indicating a possible conversion from fast to slow twitch muscle fibers. The innervated muscle profile shows no change in shape compared to the denervated muscle profile; only a change in amplitude is evident.

Table 1. 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 $n = 5$	$0.28 \pm 0.05^*$ $n = 4$
T_{peak} (ms)	39.8 ± 1.46 $n = 10$	39.2 ± 2.59 $n = 5$	$56.8 \pm 6.24^*$ $n = 4$
$T_{1/2R}$ (ms)	25.9 ± 2.07 $n = 10$	27.88 ± 1.29 $n = 5$	$48.95 \pm 6.91^*$ $n = 4$

Note: Numbers represent mean values \pm standard error. *Denotes significant difference compared to the control group, $p < 0.05$. $n =$ the number of muscles analyzed in each group.

increased to more than double the control value, the change did not reach statistical significance.

Denervation for one month resulted in considerable loss of muscle weight with both groups having less than 50% of their contralateral muscle weight. However, the stimulated muscle group lost significantly less weight than the untreated group. Figure 5 shows that this loss was due primarily to a decrease in muscle fiber cross-sectional area rather than a decrease in the number of fibers. This is also confirmed by data in Table 2, which shows mean cross-sectional areas for the three groups. The average denervated fiber cross-section was less than 25% of the control values and significantly less than the stimulated group's (36%). The ratio of stimulated to unstimulated cross sections, 1.5, was close to the ratio of the whole-muscle weights, 1.3.

4. Discussion

Traditionally, denervated muscle studies conducted on animals utilize 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.

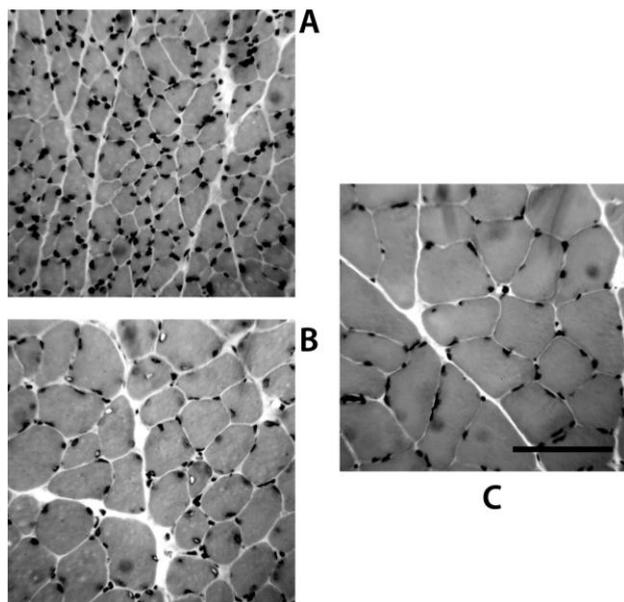


Figure 5. Transverse sections of the medial gastrocnemius muscle stained with hematoxylin and eosin (H&E). Sections were taken from the belly of the muscle. (A) denervated muscle; (B) denervated and stimulated muscle; (C) fully innervated muscle. Bar represents 100 μm and is applicable to all three panels.

Table 2. Muscle fiber area.

	Control	Denervated	Denervated-stimulated
Area (μm^2)	2605 \pm 45.7 \dagger	618.7 \pm 7.39	949.6 \pm 12.09*
	n = 489	n = 1297	n = 1508

Note: Numbers represent mean values \pm SEM. *Denotes significant difference compared to the denervated group, $p < 0.05$. \dagger Denotes significant differences compared to denervated and stimulated groups, $p < 0.05$. n = the number of fibers counted. Each group consisted of 3 animals.

The stimulator could easily produce the necessary amplitudes to elicit strong contractions in denervated muscle. Although only biphasic 400- μs pulses were used in the tests described in this paper, the stimulus output is completely flexible, allowing the researcher to select any pulse shape, duration and repetition pattern. Further, designing the system to reliably stimulate up to five animals concurrently, increased laboratory efficiency considerably.

These data demonstrate that our stimulus paradigm is effective at increasing muscle weight, twitch force, and muscle fiber cross-sectional area. However, our twitch force and muscle weight values did not approach those values reported by others. Ashley et al. [15] used a similar number of pulses in a one-hour stimulation protocol with force and weight values much closer to control values. However, their studies were done on rabbit tibialis anterior muscle using very long stimulus pulses, and stimulation began after 10 weeks of denervation. As the period between denervation and stimulation lengthens, the opportunity for recovery decreases [6] as muscle atrophies [4]. Our studies began stimulation 2 days post denervation, similar to several other studies [8,12,23]. Although Dow et al. [12] also used 100-Hz stimulation bursts, spread over 24 hours, their results showed force and weight values exceeding those of controls. It may be that the amount of rest between contractions is an important factor in maintaining muscle weight and force

[23], and a high-frequency contraction every six seconds may be too fast to maintain these properties. However, although our results did not approach control values, they did show a significant increase in muscle mass and twitch force, contrary to what Nix showed using a similar stimulation frequency in a fast twitch muscle [24]. Interestingly, the relative increase in force was much larger in our study than the relative increase in muscle mass. Other work has shown the opposite with stimulation resulting in a higher relative increase of mass than force. This may be because we elicited relatively strong contractions during stimulation. Other studies used very weak contractions throughout their stimulation protocol [12,15].

Some previous studies, using lower-limb muscles of the rat other than the gastrocnemius, showed that both contraction and half relaxation times increased in denervated muscle [8,12,15,24]. In contrast, our results show that both of these twitch characteristics in denervated rat gastrocnemius muscle were no different from those of fully innervated control muscles (Table 1). This result is supported by other studies that found that the gastrocnemius remains a fast twitch muscle following short- and long-term denervation [25,26]. As well, they found that denervated, primarily fast-twitch muscle like the gastrocnemius normally undergoes fiber type conversion from fast glycolytic to fast oxidative types (IIB to IIA) [25]. This would explain the increase in fatigue resistance in both denervated groups compared to their contralateral innervated control muscles. However, this does not explain the increase in contraction and half-relaxation times for the stimulated group. Although a stimulation frequency (100 Hz) similar to the firing frequency of fast-twitch fibers was chosen to avoid fiber type conversion, the results show that the contraction and half-relaxation times are more akin to those of slow-twitch fibers (Fig. 4). One explanation could be that chronic denervation increases the membrane time constant or the refractive period [24], resulting in a lower effective stimulation rate than the applied 100 Hz. Using a slower frequency on a fast-twitch muscle has been shown to convert fast muscle fiber types to slow types [27]. It may be that such frequent stimulation (once every six seconds in this study) at high frequencies can overwhelm the ion channel mechanisms in denervated muscle, resulting in stimulation that is similar to a slow frequency protocol.

Our one-hour stimulation paradigm was chosen because it could be translated to the clinical setting without the very high cost and invasive procedure of implanted stimulators. Of course the pulse repetition rate would be lower to match the longer twitch durations of most human skeletal muscles (e.g. 30 Hz). Clinical electrostimulation would then be delivered by removable surface electrodes, as is already done in physiotherapy clinics for muscle retraining or strengthening. Although the one-hour 20-Hz paradigm used by Ashley et al [15] resulted in better weight and force gains than our approach, it required 20-ms stimulus pulses. In earlier work [28], we investigated the use of long, low-amplitude pre-pulses in surface stimulation of intact median nerves in normal subjects, and found such long duration pulses quite painful. However, it would be worth investigating the effects of a

one-hour paradigm with stimulus rates lower than the 100 Hz used in our study (e.g. 50 Hz) but with pulse durations less than 1 ms. As well, we hypothesize that contractile input alone is not adequate to fully maintain denervated muscle, and the results of our studies fully support this.

5. Conclusions

The novel stimulator described in this paper proved very successful in both system and animal tests. Stimulus pulse parameters and repetition patterns are entered and controlled through a combination of a standard laboratory computer running a LabVIEW program and a dedicated microcontroller. This allows easy implementation and testing of new stimulus paradigms. The ability to simultaneously stimulate up to 5 animals with individual selectable pulse amplitudes is efficient, especially when large cohorts of animals are stimulated chronically over several months. The stimulation paradigm described above was effective in partially preserving muscle weight and force, compared to the untreated denervated muscle, with some results requiring further investigation. 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.

Acknowledgments

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