

## The effect of high-frequency neuromuscular electrical stimulation training on skeletal muscle properties in mice

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**Received:** September 25, 2016; **Revised:** October 15, 2016; **Accepted:** October 18, 2016; **Published online:** November 11, 2016

**Abstract:** The aim of this study was to analyze the effects of high-frequency neuromuscular electrical stimulation training (NMES) on the structure, function and oxidative capacity of the skeletal muscle using a mice model (C57BL/6J) strain, n=8). The left *tibialis anterior* muscle in mice was electro-stimulated (ST) whereas the right muscle was maintained as an internal control (CT). The ST limb was submitted to eight surface (100 Hz) NMES sessions in two weeks, with a minimum gap of 24 h between sessions. NMES training increased muscle mass ( $42.0 \pm 3.3$  vs.  $36.1 \pm 5.4$  mg,  $p < 0.05$ , effect size [ES]  $r = 0.55$ ), the mean fiber cross-sectional area (FCSA) ( $3318 \pm 333$  vs.  $2577 \pm 405$   $\mu\text{m}^2$ ,  $p < 0.001$ , ES=0.71), maximal force ( $224.7 \pm 13.8$  vs.  $184.5 \pm 30.9$  mN,  $p < 0.01$ , ES=0.64), and the rate of force development ( $1.63 \pm 0.14$  vs.  $1.34 \pm 0.20$  mN/ms,  $p < 0.05$ , ES=0.64), with no effects on the muscle oxidative profile. These results demonstrate that surface NMES induced muscle hypertrophy and instigated an improvement in the contractile properties of the TA muscle in mice. Therefore, this animal model appears to be suitable for the study of hypertrophic processes as it enables better control of the stimulus properties (intensity, duration, frequency, etc.) than other traditionally used animal models and does not require negative reinforcements or surgical procedures.

**Key words:** skeletal muscle; neuromuscular electrical stimulation; muscle mass; hypertrophy; muscle force

### INTRODUCTION

One of the main characteristics of skeletal muscle is its plasticity, as it can adapt to several stimuli, such as training or injuries, but also atrophy with aging or during prolonged immobilization periods [1]. Muscle mass plays a central role in the prevention of metabolic diseases by increasing the resting metabolic rate [2] and improving carbohydrate metabolism and insulin sensitivity [3]. In addition, recent evidence indicates that contracting muscle works as an endocrine organ that releases into the bloodstream certain molecules (myokines) with antiinflammatory effects, which exert several relevant effects in the organism [4], such as slowing cancer cell growth or protecting against chronic health problems caused by cardiovascular diseases and type II diabetes [5,6].

The role of high-frequency NMES as a “resistance exercise-like simulator” has been widely studied in recent years, as it activates the same hypertrophic signaling pathways (mammalian target of rapamycin,

mTOR) as resistance training [7], and enables the activation of type II fibers without the necessity of high intensity exercise through a non-selective and temporally synchronic activation of muscle fibers [8]. This tool has been shown to be effective in the prevention of muscle atrophy in populations with difficulties in performing volitional exercise, such as the elderly [9], during immobilization periods [10-12], and in individuals recovering from injuries [13,14].

Several animal models have been traditionally used to study the physiological mechanisms inherent to the hypertrophic process [15,16]. However, most of these models have several disadvantages as voluntary methods require negative reinforcements in order to stimulate the animals to perform the exercise, whereas involuntary methods usually require surgical procedures. Therefore, surface high-frequency NMES could be useful in basic research since it allows precise control of the stimulus variables (volume, intensity, frequency, etc.) and does not require negative reinforcement or surgical procedures.

The aim of our study was to establish whether a protocol of surface high-frequency NMES could be a valid tool to increase skeletal muscle mass and force development in mice, and would therefore be a suitable method for inducing resistance training-like adaptations, and if it could be used to study hypertrophic mechanisms.

## MATERIALS AND METHODS

A total of 8 adult and middle-aged male mice of the C57BL/6J strain (6.4±3.8 months, 25.4±3.3 g) were included in the present study. The animals were housed in ventilated racks with independent jails and a light/dark cycle of 12:12 h, temperature of 21±1°C and relative air humidity of 55±10% (means±standard error). Food (Panlab A04 rat/mouse feed) and water were provided *ad libitum*. All the processes carried out in this study were accepted by the University of Alcalá Animal Research and Experimentation Ethics Committee and complied with the European Guidelines on Laboratory Animal Care.

### NMES training

Mice were unilaterally submitted to 8 sessions of high-frequency NMES training during two weeks. The left limb was electro-stimulated (ST, n=8) and the right limb served as an internal control (CT, n=8). The animals were anesthetized with inhaled isoflurane and the ST limb was shaved and placed in a supine position with a knee and hip flexion of 90°. For the stimulation of the *tibialis anterior* (TA), two surface electrodes were placed over the animal's deep fibular nerve, located anterior to the fibular head, after the application of a conductance gel to improve the contact of the electrodes. The correct placement of the electrodes was confirmed when the stimulation elicited full ankle dorsiflexion and extension of the digits.

The high-frequency NMES training protocol used in this study was like that used by previous authors [17-19]. The ST limb was trained evoking five sets of five contractions of 5 s (pulse width 3 ms, voltage 20 V, frequency 70 Hz). A rest of 5 s was left between contractions and a 5-min rest was left between sets. The CT limb was maintained inactive during the whole protocol. The animals were submitted to four sessions per week with a minimum gap of 24 h between them.

### Analysis of contractile properties

Forty-eight h after the last NMES session the animals were anesthetized by an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), and placed in a supine position with a knee and hip flexion of 90°. The foot of the animal was tied to a force transducer (TRI201, Letica Scientific Instruments, Spain) with surgical thread. The optimal initial length of the muscle, defined as the tension that enabled a higher twitch contraction, was assessed (~40-60 mN) and kept steady for all the measurements of that muscle. Torque signals were collected continuously using a 16-bit analog-to-digital converter (PowerLab/16SP; AD Instruments, UK) and analyzed using Power Lab Chart 5 Software (AD Instruments, UK).

The contraction of the TA was evoked by percutaneously electro-stimulating the common peroneal nerve with two needle electrodes. Again, to ensure that the common peroneal nerve was being stimulated, the activation of both the TA and the *extensor digitorum longus* (EDL) was checked. The maximal force evoking a tetanic contraction with a train of ten high-frequency stimuli (pulse width 3 ms, voltage 20 V, frequency 70 Hz) was measured. This protocol was repeated three times with a 3-min gap between them and the contraction that provoked the greater peak tetanic force (termed as maximal force) was analyzed. The time to peak force and the slope of the force/time curve at 50% of the maximal tetanic force (rate of force development, RFD) were also evaluated.

### Structural and histological analysis

Once muscle force testing was complete, the animals were killed by cervical dislocation and both TA were dissected out and weighed with a precision of 0.001 g. The muscles were submerged in pre-cooled 3-methylbutane as a cryoprotective substance and finally frozen in liquid nitrogen and kept at -80°C until their posterior histochemical analysis.

Serial transverse muscle samples (10 µm) were obtained in a cryostat (Leica CM1950, Germany) at -22°C, placed in a slide and fixed with a formalin-sucrose buffer. Muscle cross sections were treated for histochemical succinate dehydrogenase (SDH) stain-

ing and immunohistochemistry [20,21]. The immunofluorescence was performed with primary anti-slow myosin heavy chain (MHC) antibodies (Abcam, United Kingdom), followed by secondary Cy3-coupled goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, USA). Individual images of the slides were taken with a 20x optical zoom across the entire cross section with a camera (Olympus DP71, Japan) attached to a microscope (Olympus BX61, Japan). The images were then assembled into a composite panoramic image (Fig. 1A and 1B) with Adobe Photoshop CC Software (Adobe Systems, California, USA).

Microphotographs (Fig. 1C and 1D) including an area of 0.59 mm<sup>2</sup> from the anterior part of the TA were taken and the FCSA of each SDH microphotograph (213±47 fibers per muscle) were measured with ImageJ Software (1.46r ImageJ, National Institutes of Health, USA). This area was chosen due to the predominance of type II muscle fibers [21], which have been reported to be more responsive to NMES training [22]. The muscle fibers were divided according to their SDH activity into aerobic (strong purple), intermediate (pale purple) and anaerobic (not stained); the oxidative capacity of the muscle was determined by counting the number of aerobic muscle fibers versus the total number of fibers.

### Statistical analysis

The normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene test) of the data were checked prior to the statistical treatment. Paired t-tests were used to make a comparison between the CT and the ST limbs. An alpha level of  $p < 0.05$  was established as the minimal level of significance. Effect sizes (Pearson's correlation coefficient,  $r$ ) were calculated to determine the magnitude of the differences [23]. All data are expressed as means±SD. The Statgraphics Plus 5.1 software (Statgraphics, Spain) was used for statistical analysis.

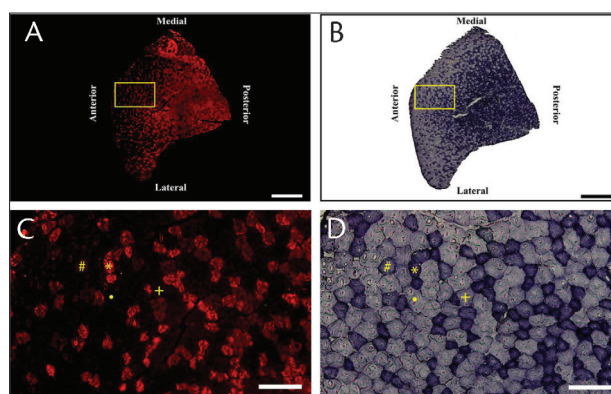
## RESULTS

A representative histological section in which the slow/fast and aerobic/anaerobic phenotype of the muscle fibers can be differentiated is shown in Fig.

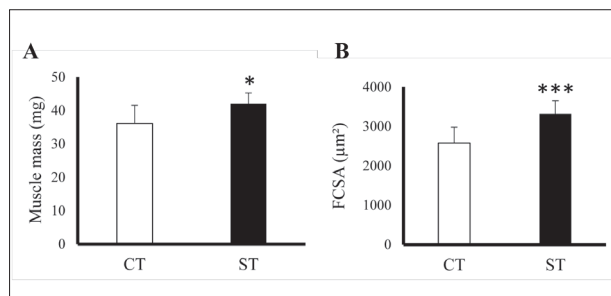
1A and 1B, respectively. A predominance of fast fibers was found in the analyzed area (Fig. 1C), in which we also detected a majority of anaerobic fibers (53.5±8.1%) in comparison with aerobic (21.2±6.4%) and intermediate (25.3±8.0%) ones (Fig. 1D).

Eight sessions of high-frequency NMES training elicited a marked hypertrophy of the TA muscle in all analyzed mice (Fig. 2), with a significant increment of the muscle mass ( $p < 0.05$ ,  $r = 0.55$ , Fig. 2A) and FCSA ( $p < 0.001$ ,  $r = 0.71$ , Fig. 2B) in the ST limbs.

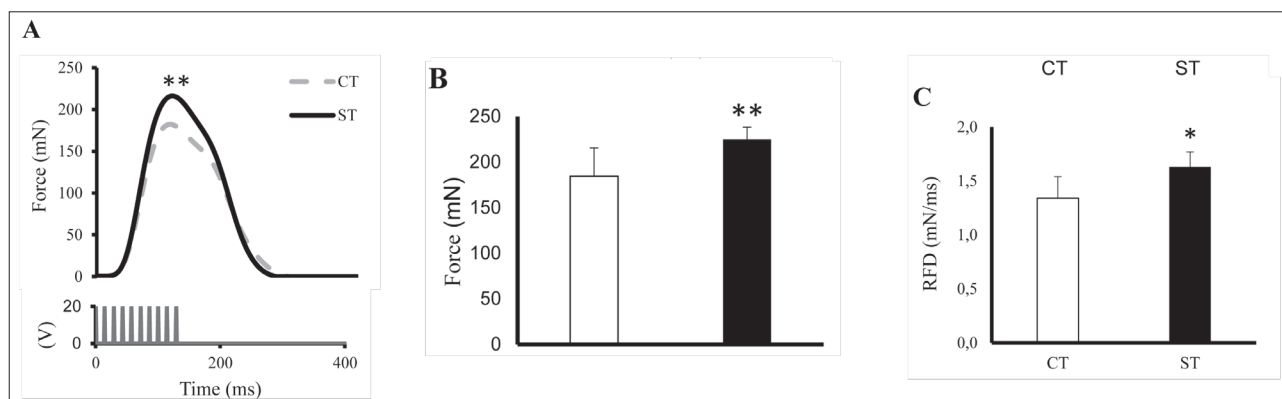
An improvement in contractile properties was also clearly observed after NMES training (Fig. 3A), with a significant increment in maximal force ( $p < 0.01$ ,



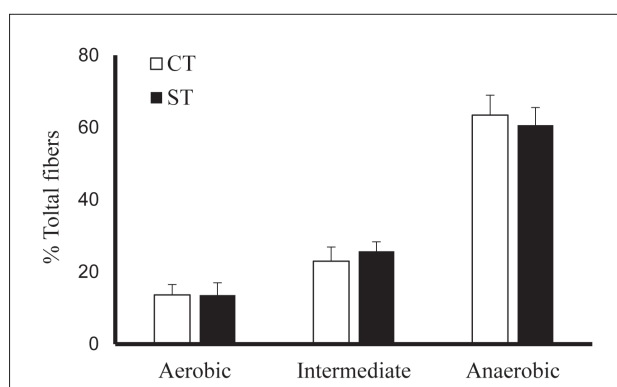
**Fig. 1.** Representative transversal cross sections of the *Tibialis Anterior* and microphotographs of the analyzed area (marked with a yellow rectangle). Serial cross sections were treated by immunohistochemistry (A) and enzyme histochemistry (B) so as to differentiate fibers into slow (red) or fast (not labeled) with regard to their myosin heavy chain composition (C), as well as into anaerobic (white), aerobic (dark purple) or intermediate (pale purple) fibers with regard to their oxidative capacity (D). Symbols (\*, +, •) represent the same muscle fibers. The scale bar represents 1000  $\mu\text{m}$  in A and B and 200  $\mu\text{m}$  in C and D.



**Fig. 2.** Effects of neuromuscular electrostimulation training on *tibialis anterior* structural properties. Muscle mass (A) and FCSA (B) of the control (CT) and stimulated (ST) limbs. Data are means±SD. Significant differences: \* $p < 0.05$ ; \*\*\* $p < 0.001$ .



**Fig. 3.** Effects of neuromuscular electrostimulation training on *Tibialis Anterior* contractile properties. Mean force trace (A) in response to ten high-frequency stimuli (70 Hz, 3 ms, 20 V); histogram representation of the maximal force (B) and the rate of force development (C) of the control (CT) and stimulated (ST) limbs. Data are means $\pm$ SD. Significant differences: \* $p$ <0.05; \*\* $p$ <0.01.



**Fig. 4.** Effects of neuromuscular electrostimulation training on *tibialis anterior* oxidative capacity. Percentage of aerobic, intermediate and anaerobic fibers of the control (CT) and stimulated (ST) limbs. No significant differences were found between control and stimulated limbs.

$r=0.64$ , Fig. 3B) and RFD ( $p<0.05$ ,  $r=0.64$ , Fig. 3C). No differences were found between the CT and ST in the time to peak ( $115\pm 10$  and  $117\pm 8$  ms, respectively).

Regarding the oxidative capacity of the muscle fibers, there were no differences in the percentage of aerobic, intermediate and anaerobic fibers between the CT and ST limbs (Fig. 4).

## DISCUSSION

Neuromuscular electrical stimulation (NMES) has been proposed as an interesting tool for the improvement of skeletal muscle properties [24]. Our results show that 8 sessions of high-frequency NMES training induced hypertrophy of the TA muscle of mice, with a significant increase in the muscle mass and the FCSA.

An increase of the quadriceps cross-sectional area after NMES training in humans has been previously described [22,25,26]. In the case of rodents, increments in muscle mass and FCSA of a fast-twitch muscle, such as the rat's gastrocnemius, have also been reported after the application of intermittent high-frequency NMES [17,18,27-29], similarly to those observed in our study in a mixed muscle such as the TA. As muscle plasticity is dependent on the muscle fiber type, being for example type II fibers more prone to atrophy during aging [30] or to hypertrophy with NMES [22], more studies analyzing the effects of different stimulation patterns in purely slow or fast-twitch muscles are required to draw conclusions about the fiber type-dependent effects of NMES training [31].

The hypertrophy found in our study after NMES training could be both sarcoplasmic and/or sarcomeric. An increase in the cell volume (cell swelling) could be produced because of the inflammatory response to muscle damage [32] and due to the accumulation of intracellular liquid because of the increase in the cell glycogen content [29]. Sarcomeric hypertrophy could result after an increase in protein synthesis, since the NMES protocol applied in our study has been previously reported to increase anabolism through the phosphorylation of p70 ribosomal protein S6 kinase beta-1 (p70s6k) [17,18], downstream of the mTOR pathway. In addition, similarly to resistance training [33,34], NMES increases growth hormone levels as a consequence of the metabolic stress induced by the accumulation of  $H^+$  and lactate [35,36], which may also enhance the anabolic processes through the activation of mTOR [37]; some authors have not found an as-



sociation between the exercise-related acute increase in anabolic hormones and muscle hypertrophy [38].

We also observed an improvement in the contractile properties after NMES training, with an increase in the maximal tetanic force and the RFD. Benefits in the muscle contractile properties after NMES training have been previously reported in humans [22,25] and rodents. The increments observed in our study are similar to those found in mice [39] and rats [18,21]. Contractile properties are dependent on many parameters, including neural mechanisms, energy disposal and muscle intrinsic properties, such as the muscle fiber type composition, Ca<sup>2+</sup> kinetics, the myofibrillar protein content or the muscle size and architecture (pennation angle, musculotendinous stiffness, etc.) [40,41]. NMES has been shown to increase the muscle glycogen supply [29] and muscle size [18], as well as to improve the neural drive [22,25,26]. Therefore, all these mechanisms could have played a role in the observed improvement of the contractile properties.

The high-frequency NMES protocol used in our study did not elicit any change in the proportion of oxidative muscle fibers (determined as oxidative capacity). It is known that low-frequency NMES promotes endurance training-like adaptations, such as capillarization and mitochondrial biogenesis [42], through the activation of the AMP-activated protein kinase (AMPK)/peroxisome proliferator-activated receptor- $\gamma$  coactivator (PGC-1 $\alpha$ ) signaling pathway [7]. In contrast, and according to our findings, it has been shown that moderate high-frequency NMES (40 Hz) does not improve the oxidative capacity in rats [43]. Resistance training and high-frequency NMES induce the activation of the mTOR hypertrophic pathway [7,17,18] and an interference between the AMPK and mTOR signaling pathways has been reported [44]. This could explain the absence of changes in the muscle oxidative profile after high-frequency NMES training.

## CONCLUSIONS

Eight sessions of surface high-frequency NMES training elicited muscle hypertrophy and an improvement in the contractile properties of the *tibialis anterior* muscle in mice. NMES appears to be a suitable ani-

mal model to study hypertrophic processes as it allows for better control of the training variables (intensity, duration, frequency) than other models, and does not require negative reinforcement or surgical procedures.

**Acknowledgements:** The authors gratefully acknowledge Javier Vicente-Tejedor, Laura Ramirez and David Rizo for their help, as well as the University of Alcalá Animal Research Center for their technical support. These experiments were financially supported by the funds of the Department of Systems Biology.

**Authors' contribution:** P.L.V., J.R.T. and P.V. conceived and designed the research. P.L.V. and A.E.M.A. performed the experiments and analyzed the data. P.L.V., J.R.T. and P.V. interpreted the results of the experiments. P.L.V. prepared the figures and drafted the manuscript. P.L.V., A.E.M.A., J.R.T. and P.V. revised the manuscript and approved the final version of manuscript.

**Conflict of interest disclosure:** No conflicts of interests, financial or otherwise, are declared by the authors.

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