Original Research

Functional Electrical Stimulation for Equine Muscle Hypertonicity: Histological Changes in Mitochondrial Density and Distribution

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ABSTRACT

Functional electrical stimulation (FES) has proven to be advantageous in reducing muscle hypertonicity and reeducating muscle memory in the human population and has recently been used in horses. Six horses ranging in age from 10 to 17 years were selected for the study. Still photographs and clinical evaluations using the Modified Ashworth Scale to characterize muscle hypertonicity were performed on all horses pre- and post-FES. Functional electrical stimulation treatments were performed over a period of 8 weeks, resulting in a total of 22 treatments per horse. Biopsies pre- and post-FES were obtained from the longissimus lumborum muscle of each horse. Mitochondrial density and distribution of the blinded samples pre- and post-FES were determined, and a two-sided Welch’s t test was used to analyze the results. The results of the clinical evaluations and of morphometric analyses comparing pre- to post-FES muscle biopsies found: (1) a significant increase ($P < .001$) in the pooled mean mitochondrial density of both glycolytic and oxidative muscle fibers, (2) a significant increase ($P < .001$) in the subsarcolemmal mitochondrial high-density patches in oxidative muscle fibers, and (3) a significant increase ($P < .001$) in the subsarcolemmal mitochondrial high-density area percentages. In summary, the clinical improvements in the reduction of hypertonicity are, conceivably, related to the daily increased muscle contraction and perfusion induced by FES training. Thus, the FES protocol used in this study produced a positive effect on mitochondrial density and distribution, which in turn may help create healthier muscle tissue that is better able to function during exercise.

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1. Introduction

Functional Electrical Stimulation (FES) is a specific type of neuromuscular electrical stimulation (NMES). Functional electrical stimulation replicates the body’s own motor neuron signal to obtain a coordinated limb or body movement rather than isolated muscle contractions obtained by other classes of NMES [1]. Therefore, improvements in articular and vertebral function are possible with FES rather than just changes in isolated muscle fibers or muscle tissue [2–11].

In horses, injury rehabilitation and performance enhancement with traditional physical therapy exercises
can be ineffective due to the difficulty in accessing deep muscles, compliance to the exercise, and the repetition required of the exercise needed to initiate a change in neuromuscular memory. Previous clinical studies have shown that the use of FES for equine rehabilitation can be beneficial to reduce muscle spasms or hypertonicity [12–15].

To obtain objective data on the effects of FES, muscle biopsy histology has been used in human research [8,16–18]. Two of the histological indicators that change, when the energy of the cell changes, are the density and distribution of mitochondria. As the myofibers require more energy, the mitochondria divide, fuse, and change shape. Because mitochondria are indicators of the plasticity of muscle tissue, the evaluation of mitochondrial characteristics could be used to determine the effects of FES on muscle at the cellular level.

Mitochondria are found in two regions: beneath the cell membrane (the subsarcolemmal mitochondria) and between myofibrils (the intermyofibrillar mitochondria). The subsarcolemmal mitochondria conceivably receive more arterial oxygen and are likely responsible for providing adenosine triphosphate (ATP) for ion and metabolite exchange and utilization. The intermyofibrillar mitochondria are inside the muscle tissue and most likely maintain the ATP supply before and during muscle contraction [19–21].

Current thought is that beneficial mitochondrial density changes are associated with active muscle movement [22]. Increases in the mitochondrial density appear to occur more frequently with endurance training rather than weight training, and the density has been shown to double with aerobic exercise [23–25]. Although mitochondrial density is lower overall in glycolytic muscle fibers when compared with oxidative muscle fibers, the change in density due to exercise is greater in glycolytic fibers than in oxidative muscle fibers [26]. In addition, aging has been shown to have a negative effect on the density and distribution of mitochondria when compared with younger muscle fibers [27–29]. An increase in mitochondrial density has been linked to improved respiratory control allowing for muscle glycogen sparing, increased intramuscular lactate clearance, and increased lipid metabolism during submaximal exercise [26,30].

Limited studies have found an increase in mitochondrial density to be a result of a FES protocol that mimics low-level exercise [18,31–33]. Recent research on humans has shown that electrical stimulation will increase the distribution of mitochondria to the subsarcolemmal area when used as an exercise protocol for aged muscle [29].

The purpose of this study was to investigate the histological changes induced by FES on equine epaxial muscle tissue through the evaluation of the density and distribution of mitochondria in muscle biopsies harvested before and after 8 weeks of FES treatments. Clinically, muscle hypertonicity will also be evaluated to determine if a reduction in hypertonicity occurred post-FES training. An improvement in the mitochondrial distribution and density post-FES would indicate that FES training produced an enhanced functional improvement in the muscle evaluated. In addition, a post-FES clinical reduction in muscle hypertonicity would show that FES training could be beneficial in reducing muscle hypertonicity. The results will provide the first objective data on mitochondrial density and distribution in the horse after a FES training regime and will also add data to previous clinical benefits of FES training [15].

2. Materials and Methods

2.1. Horse Demography

Six retired horses that had been previously used mainly for dressage riding were selected for this study. The horses ranged in age from 10 to 17 years, and veterinarians had clinically evaluated all horses for axial musculoskeletal pathologies and none had been noted. The horses had no known myogenic or neurogenic disorders and had not been tested for those pathologies. The horses had been retired 2 to 6 years, had not been ridden for at least 1 year, and were not ridden during the study. All horses were evaluated by the owner and/or trainers as being uncomfortable and tight in the back muscles and difficult to ride and therefore had been retired from riding. The owner gave informed consent to use the horses in this study. The horses were placed in a free paddock for self-exercise 1 to 6 hours daily, depending on weather conditions, and were stalled at night. No nutritional or other management changes occurred during the period of the study.

2.2. Clinical Examination of the Horses

The Modified Ashworth Scale (MAS) was used to determine the initial level of muscle hypertonicity and to grade the changes observed during the FES treatments. The details explaining the use of this scale for evaluating equine muscle hypertonicity (spasm) is documented in a previous article [34] and are outlined in Table 1. This scale is widely used to objectively evaluate the rehabilitation progress for humans and has been shown to have a 86.7% (P < .001) interrater reliability [35].

Photographs were taken on the top line of each horse before FES treatments were begun, and after all treatments were completed. The horses stood on a flat surface and

<table>
<thead>
<tr>
<th>Table 1 Modified Ashworth Scale for grading muscle spasm.</th>
</tr>
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<tbody>
<tr>
<td><strong>Grade</strong></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>1+</td>
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<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

Abbreviation: ROM, range of motion.
were positioned so that all four limbs were square and the neck of the horse was placed in a neutral position.

2.3. FES Treatments

Functional electrical stimulation treatments were given three times per week for a period of 8 weeks, yielding a total of 22 treatments per horse. The determination of the 8-week period and the number of treatments per week used for the study was based on a review of literature of exercise-induced muscle changes and the length of time needed for the observed changes to occur [36]. In addition, the number of FES treatments used in the study was in the range of those prescribed for human patients to produce a change in muscle tone [32]. The treatments were performed on the epaxial muscles of the horses including the muscle glutei and the dorsal edge of the muscle biceps femoris. During the FES treatments, the voltage was increased until pelvic rotation was obtained.

The FES system used was a 16-bit digital microcontroller and provided a pulsed, biphasic, rectangular waveform at 60 Hz, with a 0 net charge (FES310, EquiNew LLC, River Falls, WI, USA). The signal was pulsed at a rate of 2 seconds on and 2 seconds off. Three channels, with six electrodes paired in an asterisk design (Fig. 1), were used to transfer the signal to the horse for a treatment time of 35 minutes. The electrodes were placed in a pad, which was centered over the biopsy site of each horse. The skin was sponged with water, and ultrasound gel was used between the pad and the skin to reduce impedance. The voltage applied to elicit functional movement ranged from 7.6 to 15.8 volts. For each horse, the number of bursts per 35-minute treatment was 525 or 63,000 pulses (525 \times 60 \text{ Hz} \times 2 \text{ seconds}) and the total number of bursts per week was 1,575 or 189,000 pulses. After the 22-treatment protocol, the total number of pulses received by each horse was 1,386,000 for 189,000 pulses. After the 22-treatment protocol, the total number of bursts per week was 1,575 or 63,000 pulses (525 \times 2 \text{ seconds})

2.4. Muscle Biopsies

Muscle biopsies were taken from the longissimus lumborum muscle at the beginning of the study and then 8 weeks later (54 days) at the end of the study. The biopsies were taken approximately 72 hours before the first FES treatments and 72 hours after the last FES treatment. Biopsy specimens were obtained on the same side of the horse for both the pre-FES and post-FES samples. A 6-mm-diameter Bergstrom biopsy needle was used at a depth of 3 cm to obtain the muscle specimens. Two cubic centimeters of the local anesthetic lidocaine was given subcutaneously, and a 1-cm incision was made over the right longissimus lumborum muscle. Following guidelines established by Linder et al [37], the pretreatment longissimus lumborum muscle specimens were obtained 20 cm cranial to the tuber sacrale and 3 cm lateral to the midline. The posttreatment longissimus lumborum muscle specimens were obtained 18 cm cranial to the tuber sacrale and 3 cm lateral from the midline. Muscle specimens were approximately 2 cm long. One suture was used after the muscle sample was taken, and the suture was removed at 10 days. Biopsy specimens were placed on saline-moistened gauze in a plastic container and taken on ice to the laboratory within 2 hours of sampling. Fresh muscle samples were frozen in isopentane chilled in liquid nitrogen on arrival at the laboratory.

Thick sections of about 10 \mu m were stained with nicotinamide adenine dinucleotide tetrazolium reductase reaction (NADH-TR). Nicotinamide adenine dinucleotide tetrazolium reductase reaction–stained slides were photographed, and morphometry was performed on randomly selected fields in the Translational Myology Lab of the Interdepartmental Research Center (CIR-Myo) of the University of Padova, Italy. The specimens were blinded to the researcher performing the morphometry so that neither the specific horse nor the pre- or post-FES status of the sample was known. Muscle fiber size comparisons pre- and post-FES were determined in H-E, and NADH-TR–stained samples and the results of this data are discussed in a previous article [34].

Quantitative analyses of mitochondrial density and distribution in the myofibers were determined visually on microphotographs taken at medium magnification (\times20) of NADH-TR–stained sections (Fig. 2A). The mitochondria are easily identifiable in low-magnification images of the transverse sections of the muscle as very dark areas of NADH-TR stain that can be counted along the muscle fiber profiles. In the muscle cryosections from this study, the staining dots were defined well enough to discriminate larger muscle fibers with a low content of stain dots (type 2, glycolytic muscle fibers), from the smaller muscle fibers rich in staining (type 1, oxidative muscle fibers).

In the type 1 oxidative muscle fibers, a low density of intermyofibrillar-stained dots are typically distributed in a central area with a higher density of stained dots found in the subsarcolemmal coronal area. Fig. 2A shows the high-density subsarcolemmal mitochondria patches in the oxidative muscle fibers. An electron microscope image in Fig. 2B, taken from a rat, illustrates the high-density packing of mitochondria lined up in the subsarcolemmal area. No electron microscope images of the equine muscle biopsies used in this study were taken because of the preserving technique used.

2.5. Statistical Analysis

For each of the four outcomes under study (Tables 2–5), a separate hypothesis test was implemented to test the null hypothesis of an ineffective treatment against a two-sided alternative. Pretreatment and posttreatment samples were considered as representative of a wider population of
fibers, and all measurements were assumed to be independent. The null hypothesis, in each outcome, was that the FES treatment was ineffective in changing the muscle fiber characteristics. The alternative hypothesis was that the FES treatment was effective in improving muscle fiber characteristics.

Test sizes were alpha = 0.05 with power to detect a 30% relative change between pretreatment and posttreatment means equal to or exceeding 95% in all cases. Adequate power was ensured in each case by pooling muscle fiber data from each of the six horses to obtain sample sizes large enough to determine the significance difference between the predata and postdata with a high level of confidence. However, with a sample size of 6, care must be taken to not infer that the results apply to all horses of the type used in the study. The 30% value is clinically useful and conservative compared with the variation of 17.8%, 46.6%, and 67% percentual change in mean mitochondrial density due to exercise observed in previous studies on horses [38,39]. No data exist in horses to indicate what the expected mitochondrial density change due to FES would be. Power was sufficiently strong for all tests, except those specific to a few individual sample means.

Welch’s t statistic was used to gauge the significance of observed changes to means [40]. Pooled sample sizes of muscle fibers from all horses were greater than 70, and individual horse sample sizes were greater than 12. Histograms were examined and no sample distributions were found to be highly skewed, and it was determined that the assumptions for Welch’s test were adequately satisfied [41]. Reported confidence intervals are based on Welch’s t statistic.

The P values which are associated with the two-tailed Welch’s t test are reported as percent changes in the appropriate tables. In addition, standard deviations of the raw data means and standard error of the differences between the means are reported for each horse. Confidence intervals of the upper and lower limits of the absolute differences are also reported in the tables.

3. Results and Discussion

3.1. Clinical Analysis

Before FES, most horses (5/6) were initially rated by the MAS at grade 3, indicating a high level of muscle hypertonicity making spinal movement by hand manipulation impossible. A hypertonicity grade 2 was found in one of the six horses, indicating that although muscle tone was greater than normal, some joint movement was possible with manipulation. A more detailed discussion of the MAS for grading muscle hypertonicity is found in a previous article [34].

To achieve a one-grade MAS improvement in muscle hypertonicity, the horses in this study required an average of 4.3 treatments. Therefore, these horses appeared to have a somewhat higher level of epaxial muscle hypertonicity than the typical population of horses that require two FES treatments for a MAS one-grade improvement [15]. This high level of muscle hypertonicity is in agreement with the evaluations of the owner that the horses used in this study were very tight and sore in the back and thus the reason for retirement. The still photographs taken before and after 22 FES treatments showed an improvement in the degree of lordosis of the thoracic and lumbar epaxial muscles and in the symmetry of the musculature of the epaxial muscles post-FES (Fig. 3). These changes are conceivable due to the fact that FES treatment produced muscular movement, which resulted in cyclic activity of associated muscle groups in addition to the site-specific muscles that were directly stimulated.

3.2. Histological Analysis of Muscle Biopsies

Exercise in humans has been shown to increase mitochondrial density. Mitochondrial density is proportional to the oxidative capacity of the cell; therefore, high oxidative fibers show higher densities of mitochondria, although these fibers are relatively small. This improvement in
mitochondrial density can be dramatic, and one study found that after 6 weeks of endurance training, there was a total mitochondria volume increase of 50% to 60%, with close to a 100% increase in the subsarcolemmal mitochondrial [42].

In horses, limited research has been performed looking at the exercise-induced changes in mitochondrial density. One equine study found that after 4 weeks of training, a 67% increase in the overall mean mitochondrial density occurred [38]. In a later study, Tyler et al [39] found a mean pooled increase in mitochondrial volume (% of fiber area) in glycolytic fibers of 46.6% (P < .05) and an increase in oxidative fibers of 17.8% (P < .05), over a training period of 7 weeks.

In the present study, individual horse data for the glycolytic fibers showed a significant increase in mitochondrial density (P < .01) in four of the six horses, and three of the six horses showed a significant increase (P < .05) in the oxidative fibers post-FES (Table 2). As anticipated, glycolytic myofibers showed a greater overall increase in mitochondrial density when mean differences were compared pre-FES to post-FES [26].

The mitochondrial density data found post-FES from this study are similar to the results documented by Tyler et al [39]. In this study, an increase was found in the pooled mean mitochondrial density in glycolytic fibers of 43% (P < .001) and in oxidative fibers of 16% (P < .001) (Table 2). This agreement suggests that 8 weeks of FES training mimics the results of exercise training in horses. In addition, the findings in this study are in agreement with human and equine studies linking endurance training with an increase in mitochondrial density [22–27,38,39].

It is worth noting that the horses sampled for this study had been retired from training for at least 1 year; therefore, the changes noted post-FES were in untrained muscles. The total pooled percentages of oxidative and glycolytic myofibers were 64% and 36% pre-FES, respectively, and those percentages did not change post-FES. Further discussion of the muscle fiber evaluations by morphometry pre- and post-FES can be found in a previous study [34].

In several human studies, an increase in mitochondrial density was observed due to the use of electrical stimulation that mimics low-level exercise [18,29,31–33,42]. The results from this study support that data by also showing an increase in the overall mitochondrial density post-FES (Table 2).

When evaluating the changes in mitochondrial density related to age, a trend is noted that the three youngest horses showed a lower percentual change than the three older horses for both glycolytic and oxidative myofibers (Table 2). Only the youngest horse (10 years) showed a percentual decrease in the mitochondrial density of oxidative myofibers (−13.68%). However, that same horse showed a highly significant percentual increase (P < .001) in mitochondrial density of the glycolytic myofibers (25.63%). Human studies have found that aging muscle shows a reduction in mitochondrial density when compared with younger muscle, but that exercise can reverse that decline [28,29]. In this study, the percent change in mitochondrial density of the oxidative myofibers post-FES due to age shows a trend of increasing percentual
### Table 3
Comparison of pre- and post-FES intermyofibrillar and subsarcolemmal mitochondrial densities in oxidative muscle fibers stained by NADH-TR.

<table>
<thead>
<tr>
<th>Horse</th>
<th>Age</th>
<th>Mean Pre-FES Density</th>
<th>Mean Post-FES Density</th>
<th>Difference (Welch’s t Test, Degrees of Freedom [df] 95% CI)</th>
<th>Δ% (95% CI)</th>
<th>P Value (Power*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermyofibrillar mitochondrial densities</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7003</td>
<td>10</td>
<td>0.2238 (n = 13, s = .004, se = .001)</td>
<td>0.342 (n = 13, s = .008, se = .002)</td>
<td>.0195 (t = 4.31, df = 16.96) (.0053, 0.156)</td>
<td>43.87 (22.27%, 65.55%)</td>
<td>.0005 (79.24%)</td>
</tr>
<tr>
<td>7006</td>
<td>12</td>
<td>0.304 (n = 13, s = .007, se = .002)</td>
<td>0.293 (n = 13, s = .016, se = .004)</td>
<td>.0105 (t = 0.80, df = 20.44) (.0033, 0.161)</td>
<td>9.87 (~10.86%, 52.96%)</td>
<td>.4307 (63.87%)</td>
</tr>
<tr>
<td>7007</td>
<td>13</td>
<td>0.299 (n = 12, s = .005, se = .001)</td>
<td>0.127 (n = 15, s = .005, se = .004)</td>
<td>.008 (t = 0.51, df = 27.96) (.0024, 0.040)</td>
<td>6.67 (~20.17%, 33.61%)</td>
<td>.6143 (59.60%)</td>
</tr>
<tr>
<td>7005</td>
<td>15</td>
<td>0.143 (n = 14, s = .005, se = .001)</td>
<td>0.162 (n = 18, s = .005, se = .001)</td>
<td>.019 (t = 1.01, df = 27.76) (.0019, 0.055)</td>
<td>12.78 (~13.29%, 38.46%)</td>
<td>.3223 (62.31%)</td>
</tr>
<tr>
<td>7008</td>
<td>17</td>
<td>0.175 (n = 12, s = .005, se = .001)</td>
<td>0.192 (n = 12, s = .005, se = .001)</td>
<td>.017 (t = 0.84, df = 21.64) (.0024, 0.058)</td>
<td>9.52 (~13.71%, 31.14%)</td>
<td>.4088 (71.54%)</td>
</tr>
<tr>
<td>Total (pooled densities)</td>
<td>NA</td>
<td>.0196 (n = 80, s = .008, se = .001)</td>
<td>.0235 (n = 84, s = .012, se = .001)</td>
<td>.0037 (t = 2.31, df = 144.85) (.0005, 0.0069)</td>
<td>18.81 (~2.53%, 34.85%)</td>
<td>.0226 (95.45%)</td>
</tr>
</tbody>
</table>

### Table 4
Content of very high-density subsarcolemmal patches in oxidative muscle fibers stained by NADH-TR.

<table>
<thead>
<tr>
<th>Horse</th>
<th>Age</th>
<th>Mean Pre-FES Subsarcolemmal Mitochondria Patches</th>
<th>Mean Post-FES Subsarcolemmal Mitochondria Patches</th>
<th>Difference (Welch’s t Test, Degrees of Freedom [df] 95% CI)</th>
<th>Δ% (95% CI)</th>
<th>P Value (Power*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subsarcolemmal mitochondria patches</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7003</td>
<td>10</td>
<td>2.250 (n = 12, s = .622, se = .179)</td>
<td>2.583 (n = 12, s = .793, se = .179)</td>
<td>.333 (t = 1.150, df = 20.81) (.219, .385)</td>
<td>14.82 (~12.08%, 41.71%)</td>
<td>.2648 (59.38%)</td>
</tr>
<tr>
<td>7006</td>
<td>12</td>
<td>2.333 (n = 12, s = .651, se = .188)</td>
<td>2.583 (n = 12, s = .869, se = .193)</td>
<td>.250 (t = .928, df = 21.29) (.3088, .8088)</td>
<td>10.71 (~13.24%, 34.67%)</td>
<td>.3636 (69.73%)</td>
</tr>
<tr>
<td>7004</td>
<td>13</td>
<td>2.417 (n = 12, s = .515, se = .149)</td>
<td>2.667 (n = 12, s = .888, se = .256)</td>
<td>.250 (t = .844, df = 17.65) (.3732, .8732)</td>
<td>10.35 (~15.44%, 36.13%)</td>
<td>.4100 (63.25%)</td>
</tr>
<tr>
<td>7007</td>
<td>14</td>
<td>2.508 (n = 12, s = .522, se = .151)</td>
<td>3.000 (n = 12, s = .739, se = .213)</td>
<td>.500 (t = 1.915, df = 19.80) (.0450, 1.0450)</td>
<td>20.00 (~1.8%, 41.8%)</td>
<td>.0701 (77.92%)</td>
</tr>
<tr>
<td>7005</td>
<td>15</td>
<td>1.917 (n = 12, s = .289, se = .083)</td>
<td>2.417 (n = 12, s = .793, se = .229)</td>
<td>.500 (t = .928, df = 21.29) (.3088, .8088)</td>
<td>26.09 (~12.0%, 53.36%)</td>
<td>.0595 (58.33%)</td>
</tr>
<tr>
<td>7008</td>
<td>17</td>
<td>2.083 (n = 12, s = .515, se = .149)</td>
<td>2.500 (n = 12, s = .674, se = .195)</td>
<td>.417 (t = 1.701, df = 20.58) (.0933, .9266)</td>
<td>20.00 (~1.8%, 41.8%)</td>
<td>.1039 (67.84%)</td>
</tr>
<tr>
<td>Total (pooled patches)</td>
<td>NA</td>
<td>.4600 (n = 80, s = .013, se = .002)</td>
<td>.0508 (n = 80, s = .018, se = .002)</td>
<td>.0044 (t = 1.756, df = 151.338) (.0005, 0.0093)</td>
<td>10.49 (~1.20%, 23.36%)</td>
<td>.0811 (99.9%)</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; FES, functional electrical stimulation; NA, not applicable; NADH-TR, nicotinamide adenine dinucleotide tetrazolium reductase reaction; s, standard deviation; se, standard error of the mean.

* Power to detect a 30% relative change or greater, alpha = 0.05.
Table 5

| Subsarcolemmal high-mitochondrial area percentages in oxidative muscle fibers |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Horse                           | Pre-Mean Percentage | Post-Mean Percentage | Difference (Welch's t Test, Degrees of Freedom [df]) 95% CI |
| Subsarcolemmal high-density mitochondrial area (percentage) | 7003 | 12 | 74.948 (n=12, s=7.660, se=2.211) | 75.933 (n=12, s=4.136, se=1.194) | 0.984 (t=0.39, df=4.32, 6.29) | 1.31 | .7002 (99.9%) |
|                                | 7006 | 12 | 67.703 (n=12, s=5.443, se=1.794) | 75.500 (n=12, s=4.045, se=1.168) | 8.667 (t=4.37, df=20.38) | 12.97 | .0149 (99.9%) |
|                                | 7008 | 12 | 67.750 (n=12, s=1.767) | 77.000 (n=12, s=4.045, se=1.168) | 9.250 (t=4.37, df=20.38) | 12.97 | .0149 (99.9%) |
|                                | 7005 | 12 | 66.917 (n=12, s=6.215, se=1.794) | 78.583 (n=12, s=4.120) | 11.667 (t=4.37, df=19.56) | 17.44 | .0000 (99.9%) |
| Total (pooled percentages) NA | 68.434 (n=72, s=6.246, se=1.798) | 8.553 (t=5.24, df=182.42) | 12.30 | .87 |

Abbreviations: CI, confidence interval; NA, not applicable; NADH-TR, nonfluorescent tetrazolium reductase reaction; se, standard error; t, standard deviation; s, standard error of the mean.

changes when pre-FES data are compared with post-FES data as aging progresses (Table 2). Therefore, the results of this study also support the data found in previous human studies showing a reduction in mitochondrial density due to aging and the resulting improvements in mitochondrial density due to exercise of aged muscle fibers.

Mitochondria will migrate in response to exercise, and a higher mitochondrial density in the subsarcolemmal region of oxidative myofibers is one factor in determining the muscle’s oxidative capacity [43]. The intermyofibrillar mitochondria, which are more important for contractions, show less increase in density in response to endurance training [21]. In humans, a mean increase of 127% in subsarcolemmal mitochondria and an increase of 56% in intermyofibrillar mitochondria were found in sedentary older human skeletal muscle when participants engaged in a 12-week exercise program 4 to 6 times per week [44]. In addition, electrical stimulation has been shown to increase the distribution of mitochondria to the subsarcolemmal area when used as an exercise protocol [18,29].

To determine if a migration of the mitochondria occurred in this study to produce a higher post-FES subsarcolemmal in response to FES training, the densities of mitochondria in both the intermyofibrillar and subsarcolemmal areas were evaluated pre- and post-FES (Table 3). A significant increase (18.8%, P < .05) was found for the intermyofibrillar mitochondrial, and a borderline significant increase (10.5%, P = .08) was found for the subsarcolemmal mitochondrial after FES training, indicating that some mitochondrial migration was occurring.

The mitochondrial distribution results of this study seemed to indicate that a strong migration did not occur to the subsarcolemmal area post-FES, although the overall density of mitochondria increased, which warranted additional investigation. A further review of the intermyofibrillar and subsarcolemmal regions of the specimens showed that not all the subsarcolemmal mitochondrial dots could be easily counted. This is due to the fact that patches of high-density mitochondria existed, making counting individual dots in the biopsy specimens impossible, as shown in Fig. 2A. Assessment of these patches, in addition to the individual mitochondria, provided a more accurate evaluation of the total mitochondrial density changes in oxidative muscle fiber types; therefore, a counting of the mitochondrial patches was performed.

An analysis of the pooled mean data of the number of subsarcolemmal patches post-FES showed a significant increase (16.67%, P < .001) (Table 4). A significant increase in mitochondrial patches together with a significant increase in individual mitochondria density indicates a sustained increase in metabolic activity in the myonuclei. In addition, the increase in subsarcolemmal mitochondria post-FES adds to the evidence that there was a significant overall change in mitochondrial density as well as a significant change in the distribution of the mitochondria. The mitochondrial changes found in this study may be related to increased blood perfusion as an adaptation to increased contractions per day in FES-treated muscles. This increase was observed in spite of the fact that the muscle biopsies were harvested 72 hours after the last stimulation session.
To gain further insight into these findings that indicated an increase in mitochondrial density and distribution post-FES, the percentual subsarcolemmal area of the oxidative muscle fiber that contains a high density of mitochondria (coronal area) was compared with the area of the muscle fiber that contains a low density of mitochondria both pre- and post-FES. Fig. 2A illustrates the delineation of high- to low-density areas with a circle showing that the inside of the boarder contains the low-density mitochondrial area and the outside of the boarder contains the high-density mitochondrial area.

A highly significant ($P < .001$) increase of 8.7% in the pooled means of the percentual area of high-density subsarcolemmal mitochondria was found post-FES in the oxidative muscle fibers (Table 5). Individual horse data showed that four of the six horses had a significant ($P < .05$) increase in the subsarcolemmal coronal area (Fig. 4, Table 5). These data most clearly illustrate the improvement in mitochondrial density and distribution as a result of FES training due to the fact that both individual mitochondria and mitochondrial patches were included in the evaluation of the data.

Interestingly, the four oldest horses showed significant differences in the overall subsarcolemmal high-density mitochondrial area percentages, whereas the two youngest horses did not show overall significant changes post-FES training (Fig. 4, Table 5). These data again support the findings of previous studies on aged muscle showing that the changes in mitochondrial distribution and density are more dramatic than in younger muscle [28,29,44]. Also, it is noted that the four older horses had increases in mitochondria percentages that brought the post-FES subsarcolemmal high-density mitochondria percentage levels almost to the level of the pre-FES mitochondrial high-density levels of the youngest horse, which showed no significant changes post-FES.

Other human studies have concluded that the increases in mitochondrial density and changes in mitochondrial distribution could be the result of the adaptive mechanisms...
of the muscle fibers to the increased number of contractions per week and of the associated increase in muscle perfusion [31–33]. In this study, the muscles stimulated by FES were those subjectively evaluated as being hypertonic and therefore did not have the characteristics of normal, healthy muscle, which had been confirmed by a previous study looking at the muscle fiber characteristics of these same horses [34]. The total number of FES bursts was 1,575 or 189,000 pulses per week for a period of 8 weeks. Therefore, it could be assumed that the increased use of these muscles through FES could promote the contractile and metabolic properties that would increase mitochondrial density. Again, the increase in mitochondria density seems to be a retained effect (medium-term adaption) because the muscle biopsies were harvested 72 hours after the last stimulation session.

4. Conclusions

In conclusion, the results of the clinical evaluations and of the morphometric analyses comparing pre-FES to post-FES muscle biopsies found (1) a significant increase ($P<0.001$) in the pooled mean mitochondrial density of both glycolytic and oxidative muscle fibers, (2) a significant increase ($P<0.001$) in the subsarcolemmal mitochondrial high-density patches in oxidative muscle fibers, and (3) a significant increase ($P<0.001$) in the subsarcolemmal mitochondrial high-density area percentage. In summary, the clinical improvements in the reduction of hypertonicity are, conceivably, related to the daily increased muscle contraction and perfusion induced by FES training. Thus, the FES protocol used in this study produced a positive effect on mitochondrial density and distribution, which in turn may help create healthier muscle tissue that is better able to function during exercise.

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References


