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Original Article

Hylonome

Electrical impedance alterations in the rat hind limb with unloading

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Abstract

Objectives: Methods are needed for quantifying muscle deconditioning due to immobilization, aging, or spaceflight. Electrical impedance myography (EIM) is one technique that may offer easy-to-follow metrics. Here, we evaluate the time course and character of the change in single- and multi-frequency EIM parameters in the hind-limb suspension model of muscle deconditioning in rats. **Methods:** Sixty-two rats were studied with EIM during a two-week period of hind limb unloading followed by a two-week recovery period. Random subsets of animals were sacrificed at one-week time intervals to measure muscle fiber size. **Results:** Significant alterations were observed in nearly all impedance parameters. The 50 kHz phase and multi-frequency phase-slope, created by taking the slope of a line fitted to the impedance values between 100-500 kHz, appeared most sensitive to disuse atrophy, the latter decreasing by over 33.0±6.6% ($p<0.001$), a change similar to the maximum reduction in muscle fiber size. Impedance alterations, however, lagged changes in muscle fiber size. **Conclusions:** EIM is sensitive to disuse change in the rat, albeit with a delay relative to alterations in muscle fiber size. Given the rapidity and simplicity of EIM measurements, the technique could prove useful in providing a non-invasive approach to measuring disuse change in animal models and human subjects.

Keywords: Electrical Impedance Myography, Hind Limb Unloading, Disuse, Sarcopenia, Muscle Fiber

Introduction

Muscle deteriorates during prolonged bed rest and disuse, aging (sarcopenia), or prolonged weightlessness during spaceflight due to varying degrees of increased protein degradation and decreased muscle protein synthesis[1]. Such loss has a variety of potentially important consequences, including primary muscle weakness[2], physical disability[3], increased risk of osteopenia[4], and the development of impaired glucose tolerance[5]. In recent years, a complex set of cellular mechanisms underlying this deterioration has been identified[6], and approaches to forestalling or reversing muscle loss through exercise and pharmacological intervention are actively being investigated both in academia and industry[7].

Despite the clear clinical importance of such skeletal muscle deterioration, methods for easily assessing its severity remain relatively poorly developed. Simple circumferential measurements of a limb are insensitive to the presence of sarcopenia[8]. In contrast, measurement of the cross-sectional area via magnetic resonance imaging and computerized tomography are very accurate in judging the amount of atrophy[9-10]; imaging can also be quantified to evaluate the percentage of fat present in the muscle that occurs commonly in sarcopenia[8]. However, both methods are relatively costly, are inconvenient for regular clinical use, and in the case of computerized tomography, requires exposure to ionizing radiation. Dual x-ray absorptometry has also been utilized[11], as have a number of molecular biomarkers, such as insulin-like growth factor-1 or interleukin-6[12]. None has reached widespread acceptance. Standard electrophysiological tools such as electromyography are generally considered insensitive to disuse change[13].

One convenient technology that might provide a simple index of muscle health and that could be especially useful for the detection and quantification of disuse change in a variety of situations ranging from clinical care to spaceflight, is electrical impedance myography (EIM)[14]. EIM is a non-invasive,

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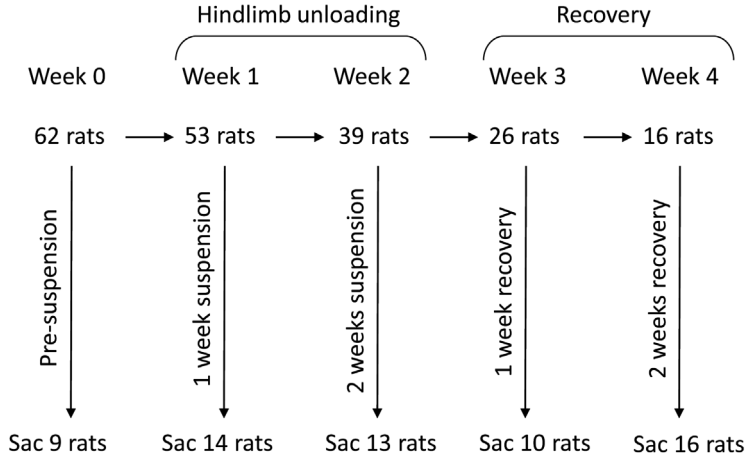

Figure 1. The flow chart of experimental design.

bioimpedance-based technique in which a weak, high-frequency electrical current is applied to a discrete region of muscle with close attention to electrode positioning and orientation, and the consequent surface voltages measured[15]. Several parameters are obtained, including the tissue's reactance, resistance, and phase angle that can provide a quantitative measure of muscle condition. In addition to showing substantial changes in primary neuromuscular diseases, such as amyotrophic lateral sclerosis and spinal muscular atrophy[16-17], EIM also may be sensitive to muscle condition due to disuse. A single study of 10 ankle-fracture patients showed low EIM phase values after 6 weeks of being non-weight bearing; these changes normalized after regular activity was resumed[18]. However, the relationship between these data and the underlying the muscle pathology was not studied. Moreover, since the individuals were not evaluated until after sustaining their fractures, EIM alterations compared to their true baseline could not be established.

In this study, we begin to assess systematically the potential value of applying EIM in the assessment of muscle deconditioning and its treatment, by applying it to measurements in the standard hind limb suspension model of disuse atrophy in rats[19].

Materials and methods

Animals

Sixty-two male Wistar rats, 14 weeks of age, were obtained from Charles River Laboratories (Wilmington, MA). Animals were allowed to acclimate at least 48 hours prior to use in any studies and were fed a regular diet *ad libitum* before, during, and after hind limb unloading studies. All studies were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center.

Animal hind limb unloading

A suspension cage was developed following the approach of Riley et al, 1990 in order to completely unload the hind limbs[19]. The suspension cage consisted of an overhead swivel and tether assembly attached to the top of a polycarbonate tub, 15" in height and 10" in diameter. This round design permitted the animal 360° rotation, relatively free movement around the cage with its fore limbs, and unlimited access to food and water. Only one animal was housed per cage. A wire was attached to a swivel; this wire was attached to the rat's dorsal, proximal tail with Benzoin tincture. Gauze and tape were also used to attach the wire to the animal securely, while ensuring that it was non-irritating.

Experimental design

As shown in Figure 1, after baseline measurements were obtained, animals were suspended for 2 weeks; at the conclusion of that time period, the animals were released from suspension, and placed back singularly in regular cages for the 2-week recovery period. Animals were also briefly removed from suspension at 1 week to obtain measurements. Nine to 16 rats were euthanized each week and the gastrocnemius muscles removed and preserved for pathologic evaluation. Any animal that became inadvertently unsuspended (e.g., due to equipment failure) for any reason during the two weeks of suspension was excluded from the entire study (the values provided in Figure 1 do not include such animals).

EIM measurements

EIM measurements were performed at baseline, at 1 week and at 2 weeks into suspension, after which the period of suspension was complete, and then at 1 and 2 weeks recovery. Each animal was returned to the regular animal holding cage to walk freely for an hour before EIM was performed in order to help reestablish normal fluid distributions in the limb.

EIM measurements were performed as previously described[20]. Briefly, under isoflurane anesthesia the rat was placed in the prone position with the left limb affixed with adhesive tape and spread at an approximately 45° angle to the

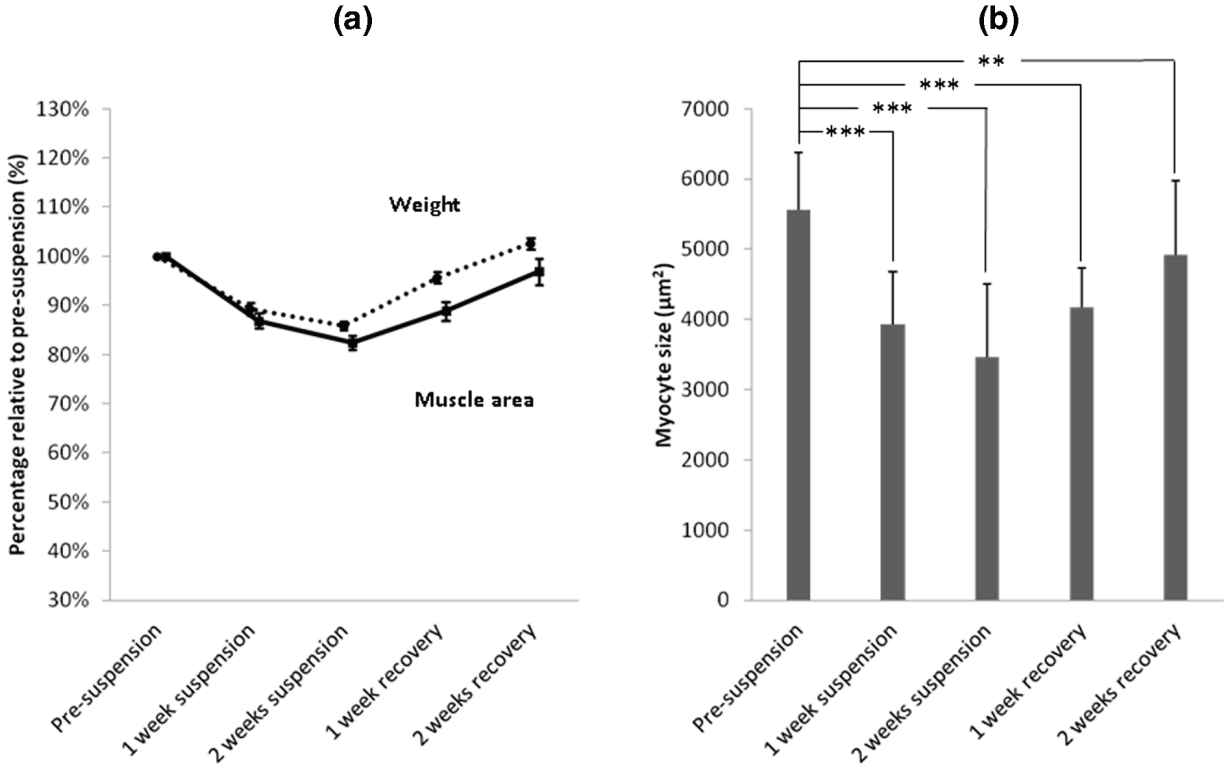

Figure 2. a. Weight and muscle cross-sectional area +/- SEM as a percentage from baseline (significance based on repeated measures ANOVA); **b.** Myocyte size +/SEM from sacrificed animals at each time point. Significance based on one-way ANOVA.

	Sample size (n)	Myocyte size (µm²)
Pre-suspension	9	5555.5±203.6
1 week suspension	14	3932.8±186.2***
2 weeks suspension	13	3455.0±262.4***
1 week recovery	10	4169.3±140.5***
2 weeks recovery	16	4923.9±263.1***

*One way ANOVA was performed. $F_{(4,57)}$= 9.938, p<0.001. Significance level as compared to pre-suspension using Bonferroni post-hoc tests are indicated by *p<0.05, **p<0.01, ***p<0.001.*

Table 1. Alterations of myocyte size (± SEM) over time with animal numbers in each group.

	Weight (g)	Muscle area (cm²)
Pre-suspension	412.2±4.7	3.0±0.1
1 week suspension	367.9±5.2***	2.6±0.0***
2 weeks suspension	353.9±5.4***	2.4±0.0***
1 week recovery	394.3±5.8**	2.6±0.1***
2 weeks recovery	422.8±6.6	2.9±0.1

*Repeated measures ANOVA was performed. For weight: $F_{(4, 60)}$= 68.066, p<0.001; for muscle area: $F_{(4, 60)}$=35.2, p<0.001 (F values include Huynh-Feldt correction). Significance levels here and in Tables 3 and 4 as compared to pre-suspension using Tukey's least-significant difference post-hoc tests are indicated by *p<.05, **p<.01, ***p<.001.*

Table 2. Alterations of weight, muscle cross-sectional area (± SEM) over time (N=16).

spine. All fur over the left calf region was removed with clippers and a depilatory agent. To ensure similar positioning of electrodes for EIM from week to week, a pinpoint tattoo was applied to the skin overlying the center of the gastrocnemius muscle at the time of the first assessment. Four adhesive electrodes (Ambu Neuroline 700 surface adhesive Ag–AgCl electrodes, Product # 70010-K/C/12, AMBU Inc., Bethesda, Maryland), cut to 18 X 3.5 mm in size, were used for EIM measurements. The electrodes were secured to the rat limb, spaced 4 mm apart, with medical adhesive tape (3 M Micropore, 3 M Health Care, St. Paul, Minnesota). The center two served as voltage electrodes and the outer two served as current-injecting electrodes. Along with animal weight, the girth of the leg at the tattoo position on the skin was also measured with a small piece of string recorded weekly to monitor the geometric changes of the leg. From this value, the cross-sectional area of the limb was approximated via a simple geometric relationship, assuming the cross-sectional area to be approximately circular.

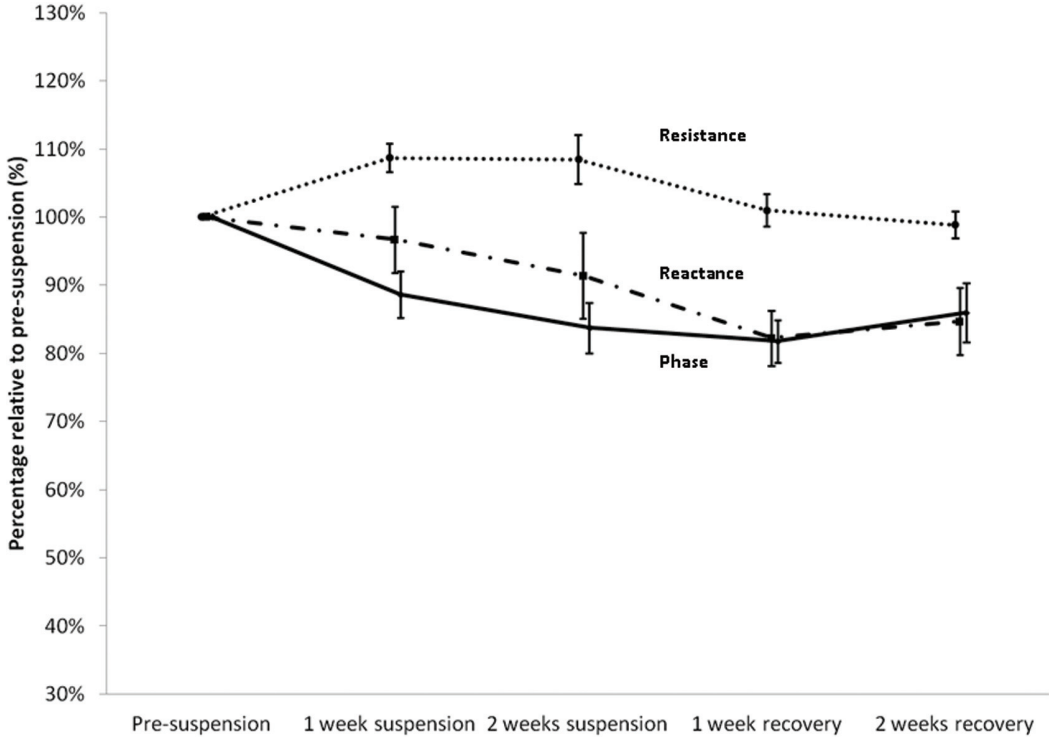

Figure 3. 50 kHz EIM measures +/-SEM over time as a change from pre-suspension. Significance based on repeated measures ANOVA.

EIM measurement system

EIM was performed using a lock-in amplifier, Signal Recovery Model 7280, Advanced Measurement Technology Inc., Oak Ridge TN coupled with a very low capacitance active probe (Model 1103 of Tektronix, Beaverton, OR) as previously described.21 Measurements were obtained over a frequency range of 3 to 500 kHz.

Quantitative pathological study

The muscle was immediately frozen in methylbutane cooled in liquid nitrogen for pathological study and stored at -80ºC until ready for use. The frozen tissue was cut in a Tissue Tek II cryostat (Miles Laboratories, Inc., Elkhart, IN) into 10 micron thick sections and stained with hematoxylin and eosin. Stereological measurements[22] were made using a Zeiss Axiophot microscope with a LUDL motorized stage interfaced with a Dell Optiflex 380 computer running Stereo Investigator (MBF Biosciences, Inc., Williston, VT) software. This software allows a non-biased quantification of fiber sizes. After the investigator sets a series of initial parameters, including the section of tissue from which to choose cells, the system automatically and randomly selects groups of cells to count. A total of approximately 300 cells were evaluated from each animal. In order to further reduce the potential for any bias, the evaluator (Andrew J. Spieker) was blinded to the state of the animal. For each slide, a histogram of cell size (in cross-sectional area and diameter) was obtained.

	Resistance (Ω)	Reactance (Ω)	Phase (º)
Pre-suspension	74.1±1.0	21.7±0.4	16.3±0.3
1 week suspension	80.5±1.9**	21.0±1.2	14.5±0.5*
2 weeks suspension	80.3±2.2	19.8±1.3	13.7±0.6**
1 week recovery	74.8±1.9	17.8±1.0**	13.4±0.6***
2 weeks recovery	73.2±1.2	18.4±0.9	14.0±0.6*

Repeated measures ANOVA was performed. Resistance: $F_{(4, 60)}=4.828$, $p=0.002$; Reactance: $F_{(4, 60)}=2.96$, $p=0.027$; Phase: $F_{(4, 60)}=6.05$, $p<0.001$ (including Huynh-Feldt correction).

Table 3. Alterations in single frequency, 50 kHz resistance, reactance, and phase (± SEM) over time (N=16).

Data analysis

Resistance *(R)* and reactance *(X)* were extracted at 50 kHz and phase *(θ)* was calculated via the equation $\theta=\arctan(X/R)$. Collapsed multi-frequency EIM parameters, including the log-resistance-slope, reactance-slope, and phase-slope, were calculated by taking the negative of the slope of a linear fit of the data across a pre-specified frequency range as previously described[23]. Based on the parametric nature of the resulting data, normality was assumed for all statistical tests. ANOVA (with a Bonferroni correction for post-hoc two-group comparisons) was used for analyses involving the subsets of sacrificed animals at different time points; repeated measures ANOVA (with

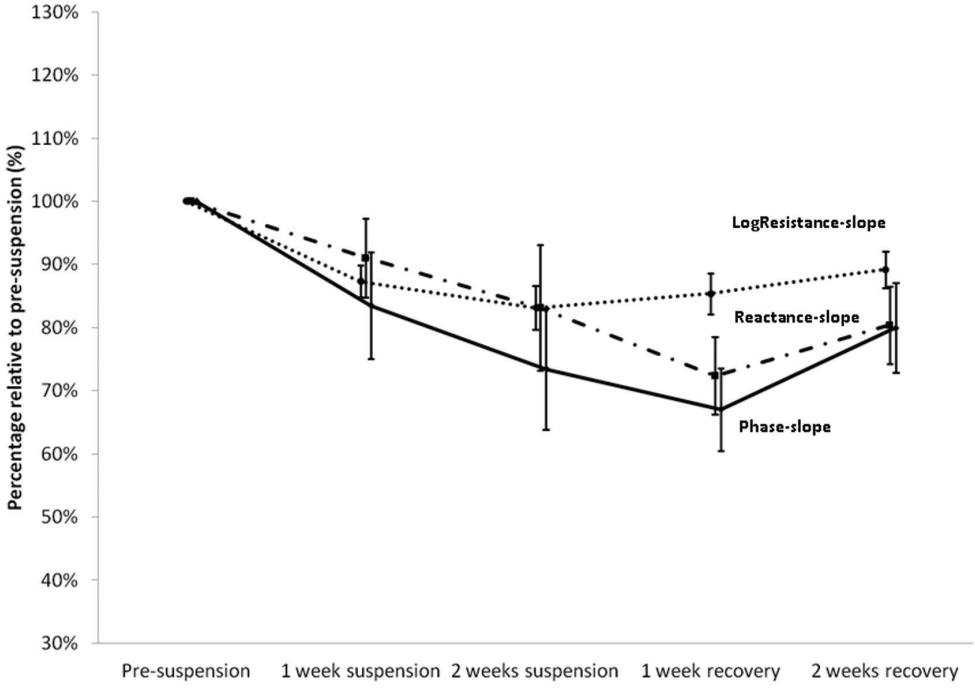

Figure 4. Multifrequency EIM measures +/SEM over time as a change from pre-suspension. Significance based on repeated measures ANOVA.

	Log Resistance-slope (Ω/kHz)	Reactance-slope (Ω/kHz)	Phase-slope (°/kHz)
Pre-suspension	14.1±0.4 x 10^{-2}	21.0±0.6 x 10^{-3}	13.3±0.6 x 10^{-3}
1 week suspension	12.3±0.3 x 10^{-2}**	19.1±1.4 x 10^{-3}	11.1±1.1 x 10^{-3}
2 weeks suspension	11.7±0.5 x 10^{-2}**	17.4±1.8 x 10^{-3}	9.8±1.0 x 10^{-3}
1 week recovery	12.0±0.3 x 10^{-2}**	15.2±1.2 x 10^{-3}**	8.9±0.8 x 10^{-3}**
2 weeks recovery	12.6±0.4 x 10^{-2}	16.9±1.2 x 10^{-3}	10.7±0.9 x 10^{-3}

Repeated measures ANOVA was performed. Log-resistance-slope: $F_{(4, 60)}=8.26$, $p<0.001$; Reactance-slope: $F_{(4, 60)}=2.84$, $p=0.032$; Phase-slope: $F_{(4, 60)}=3.39$, $p=0.014$ (including Huynh-Feldt correction).

Table 4. Alterations of EIM multifrequency measures (± SEM) over time (N=16).

Tukey's least-significant difference for post-hoc two-group comparisons) was used to assess the alterations of EIM measurements over time in the sixteen animals completing the study, with Huynh-Feldt correction for sphericity. Statistical analyses were performed with SPSS (SPSS, Inc, Chicago).

Results

Weight, limb cross-sectional area, and myocyte size

Animal weight and limb cross-sectional area showed substantial reductions with hind limb suspension, both of which completely reversed during the two-week recovery period (Tables 1, 2 and Figure 2). Myocyte size, in contrast, remained significantly reduced even after 2 weeks recovery (p=0.001), being on average about 11.4±9.1% lower than baseline. Changes in myocyte size were also greater than changes in the overall cross-sectional area of the limb. For example, after two weeks of suspension, myocyte size decreased nearly 37.8±9.5% reduction, whereas the cross-sectional area of the limb decreased by only 17.6±1.4%.

50 kHz EIM alterations

As shown in Table 3 and Figure 3, there were significant changes in the single frequency, 50 kHz EIM parameters for resistance, reactance, and phase during the period of suspension and recovery. For the most part, the EIM data mirrored the changes in muscle fiber size and cross-sectional area showing changes during the period of suspension that appeared to reverse for the most part after the animals were returned to normal activity, although

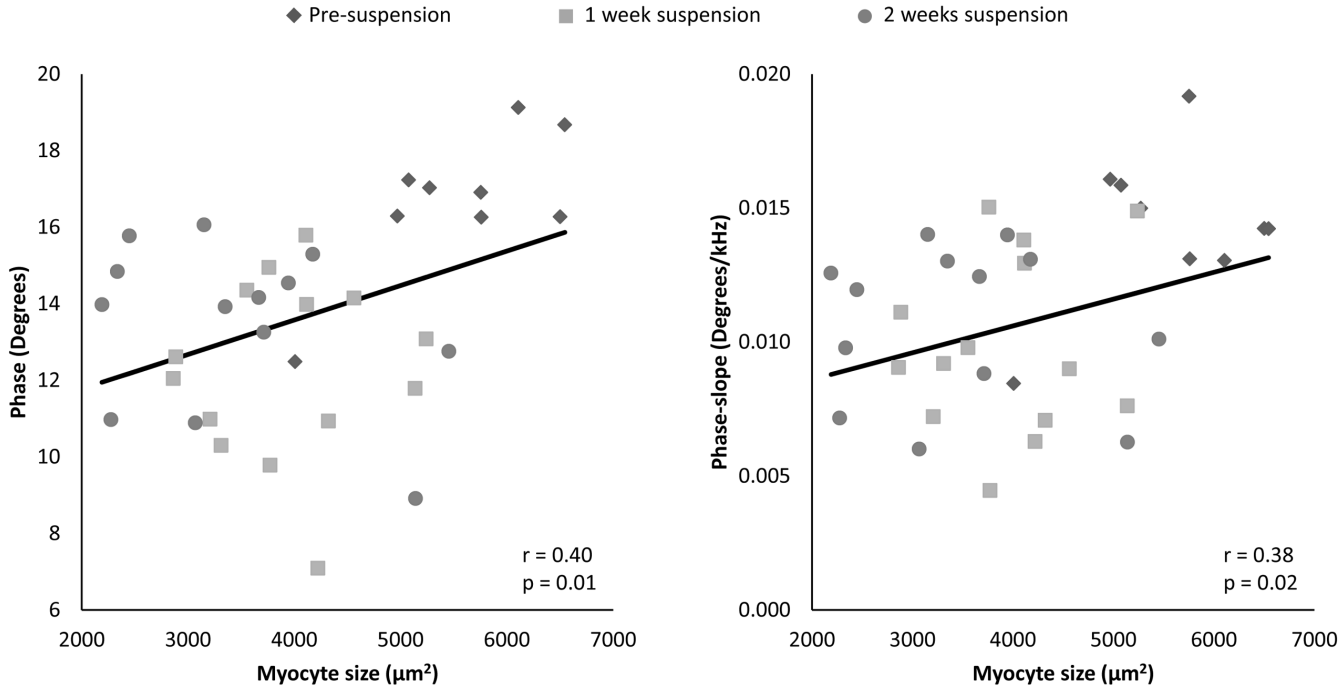

Figure 5. Correlation between muscle fiber size and two EIM measures (50 kHz phase and multifrequency phase slope). Significance based on Pearson correlation.

with a delay. Phase reduction after two weeks of suspension was 16.2±3.7%, and after two weeks recovery still was 14.0±4.3% below baseline. As demonstrated in the figure, the changes in the 50 kHz EIM data were similar in magnitude to changes in the weight and limb cross-sectional areas, but considerably smaller than those for muscle fiber size described above.

Multifrequency EIM alterations

All three multifrequency measurements showed much larger changes on average than did the single frequency data (Table 4, Figure 4), with each measure dropping at least 15%. However, the greater variability in the measurements also resulted in fewer of the changes reaching significance. Of the three measures, the phase-slope showed the greatest change, with its maximal decline reaching 33.0±6.6% ($p<0.001$). Like the 50 kHz data, there was a lag in the maximal change in values, with the greatest change occurring at approximately 1 week of recovery rather than immediately after 2 weeks of suspension.

Relationship between EIM alterations and muscle fiber size

Figure 5 shows the relationship between muscle fiber size and the impedance alterations for both 50 kHz phase and the multifrequency EIM phase value.

Discussion

This study demonstrates significant alterations in the EIM values that occur during two weeks of hind limb unloading that do not fully reverse during two weeks of recovery. In addition, of all the EIM parameters, the phase parameters, both 50 kHz and multifrequency values, showed the greatest sensitivity to change and would likely serve as the most useful measures of muscle status when assessing disuse. While gross morphological assessment (limb cross-sectional area) showed a return to baseline across the animals, muscle fiber size actually remained about 11.4±9.1% lower than baseline (a significant difference), consistent with work by others suggesting that longer time periods of recovery are needed to achieve a full return to baseline[24]. Taken together, these data suggest that EIM can serve as a useful measure of disuse effects on muscle and may provide an indirect index of the degree of muscle fiber atrophy, although further study is needed to better understand the exact time course of change.

Two fundamental questions remain: what is the mechanism of the impedance change and what accounts for the delay observed in most impedance parameters? The observed reduction in muscle fiber size most likely explains this impedance change, since a reduction in muscle fiber size reduces the charge storage capacity of the tissue. These alterations in membrane capacitance will have the greatest impact on the reactance and phase values. It is also possible that other compositional changes occur in the muscle during this period of time, such as the deposition of increased connective tissue or fat[25]. Indeed, other factors beyond simple changes in cell size must be at play since the correlation between the EIM values and cell size is relatively modest, with R values of approximately 0.4 (thus explaining only 16% of the observed

variance). Of note, at these relatively low frequencies, it is unlikely that any major *intra*cellular changes are contributing to the observed alterations in the impedance data.

As for the second question, additional structural alterations to the tissue upon reloading could provide a simple explanation. For example, actual myocyte membrane rupture[26] and muscle inflammation[27] have been well described to occur shortly after reloading. However, we did not specifically assess these on pathological evaluation, our main interest being in the determination of muscle fiber size. Moreover, the time course of such changes would demand a different experimental design than used here with planned animal sacrifice over a period of several days after reloading.

Based on finite element modeling data [unpublished results] it is likely that much of the electrical current flow in EIM is passing through the most superficial layers of the gastrocnemius muscle, which in the rat consists mainly of Type 2 fibers. Thus, it is likely that only alterations in these muscle fibers are being identified. However, Type 1 fibers of the deep gastrocnemius muscle and soleus muscles typically undergo the greatest change during hind limb suspension[28]. Thus, the alterations being measured here using EIM are possibly conservative in their estimation. It is theoretically possible to reorient the electrodes such that deeper regions of muscle are interrogated by the electrical current, and this may be pursued in future work. However, it is also worth considering that the changes in Type 1 vs. Type 2 fibers during animal suspension are different from those observed in human subjects, in whom Type 2 fiber atrophy is usually more prominent especially during spaceflight[29]. Thus, the observed differences here are perhaps especially relevant to changes that occur in human muscle with unloading.

There are several limitations to this study. First, it is clear that the animals had not fully recovered after two weeks. Extending follow up for several more weeks post-suspension is indicated to determine if and when the values eventually return to baseline. Also, as previously noted, more frequent measurement and pathological assessment in the several days post-suspension may provide additional insight into the time course of changes. Third, we did not assess food consumption and it is possible that this could play a role in the loss of muscle tissue as well. Fourth, in our analyses, we did not attempt to correlate specific fiber type loss with the alterations in the EIM data, although as noted above, most of the changes are most likely related to Type 2 fiber atrophy in the most superficial layers of the muscle. Fifth, in this analysis we assessed EIM data only in the longitudinal direction (i.e., with current flow along the muscle fibers); changes in the directional dependence of the EIM data may also be important and study with electrical current orthogonal to the fibers could provide additional important metrics. Finally, we cannot exclude the possibility that some fluid shifts impacted the data at the time the animals were removed from suspension; however, such an effect would clearly not explain the persistence of alterations in the EIM data even after 2 weeks of recovery.

In summary, we have shown that EIM surface measurements can provide a means of assessing muscle change caused by disuse and that of all the measures, the phase and multi-frequency phase-slope metrics appear most sensitive to such change. Further study, however, is needed to better understand the time course of EIM change and its relationship to muscle histopathology, especially given the delay in EIM alterations. Nonetheless, EIM thus offers promise as a convenient tool in the evaluation of muscle loss in a variety of disorders including in sarcopenia, critical illness, cachexia, and spaceflight, especially since, unlike standard approaches such as magnetic resonance imaging, computerized tomography and dual x-ray absorptometry, portable, lightweight versions of the technology will soon be available[30].

Acknowledgement

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