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Estimating myofiber size with electrical impedance myography: a study in ALS mice

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Abstract

Introduction—A method for quantifying myofiber size noninvasively would find wide use including primary diagnosis and evaluating response to therapy.

Methods—Using prediction algorithms, including the least absolute shrinkage and selection operator (LASSO), we applied multifrequency electrical impedance myography (EIM) to amyotrophic lateral sclerosis (ALS) SOD1 G93A mice of different ages, and assessed myofiber size histologically.

Results—The multifrequency EIM data provided highly accurate predictions of myofiber size with a root mean squared error (RMSE) of only 14% in mean myofiber area (corresponding to $\pm 207 \mu\text{m}^2$ for a mean area of $1488 \mu\text{m}^2$) and a RMSE of only 8.8% in predicting the coefficient of variation (CoV) in fiber size distribution.

Discussion—This impedance-based approach provides predictive parameters to assess myofiber size and distribution with good accuracy, particularly in diseases in which myofiber atrophy is the predominant histological feature, without the need for biopsy or burdensome quantification.

Keywords

myofiber size; electrical impedance myography; prediction; mouse; phase; reactance; resistance

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INTRODUCTION

The assessment of myofiber cross-sectional area and the degree of cellular atrophy is one of the most basic aspects of muscle histological analysis. Atrophy can take many forms, including group atrophy associated with denervation and reinnervation, atrophy associated with a primary myopathic process, or atrophy associated with disuse or corticosteroid use.¹ Whereas each of these types of atrophy is distinctly different, they all share the common feature of representing some form of pathological alteration of tissue. To date, assessing such muscle pathology is not possible without performing a biopsy and removing a piece of tissue for microscopic analysis. While standard radiological and electrophysiological techniques are not capable of assessing myofiber size, one technique that holds promise for this specific use is electrical impedance myography (EIM). In EIM, a weak electrical current is applied to a region of muscle and the consequent voltages are measured from a second set of electrodes.² The alterations in the recorded voltages, including changes in the amplitude of the voltage and the timing relative to the applied current, are closely related to the compositional and morphological characteristics of the tissue.

Recently, we evaluated the prospect of using EIM specifically for the assessment of myofiber size by evaluating a group of wild-type mice of varying age from post-natal day 5 to post-natal day 35, a period of dramatic myofiber growth.³ Using four needle electrodes to obtain multifrequency EIM measurements and a standard multiple regression prediction technique termed LASSO (least absolute shrinkage and selection operator)⁴ we were able to demonstrate our ability to predict myofiber size with errors as low as 12% based on the impedance data and age of the animal alone.

In this study we sought to advance this approach by evaluating pathological tissue, performing the measurements using surface rather than needle electrodes, and using EIM parameters alone in the regression equation. To do so, we performed EIM on a cohort of ALS SOD1 G93A mice⁵ of various ages and then euthanized the animals to evaluate quantitative histopathology, with the goal of determining how precisely we could predict myofiber size in this disease model. Ultimately, if successful, this basic EIM approach could be used for quantifying the degree of fiber atrophy and approximating mean myofiber size and the degree of denervation in a given muscle in patients with a variety of neuromuscular disorders.

METHODS

Mice

Beth Israel Deaconess Institutional Animal Care and Use Committee approval was obtained prior to the initiation of any studies. Breeding pairs of ALS (B6SJL-Tg(SOD1-G93A)1Gur/J) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and bred to obtain 37 animals (approximately half female and half male). In order to study varying fiber size, animals were euthanized at various ages ranging from 8–18 weeks (approximately 6–7 animals per fortnight, at 8, 10, 12, 14, 16, and 18 weeks).

Electrical impedance myography (EIM)

Surface impedance measurements were performed under 1–2% inhaled isoflurane anesthesia delivered by nose cone with body and muscle temperature being maintained by a heating pad (37°C), as previously described ⁶. After removing the fur with clippers, a depilatory agent was applied to the left hind limb to eliminate any remaining fur, and the skin was cleaned with 0.9% saline solution. The animal was placed in a prone position and both legs were taped to the measuring surface at an approximately 45° angle extending out from the body in preparation for measurements. A fixed rigid 4-electrode impedance-measuring surface array was applied over the left gastrocnemius muscle. EIM measurements were performed with the mView system (Myolex, Inc, San Francisco, CA), which obtains impedance data at 41 frequencies from 1 kHz to 10 MHz as previously described ^{6,7}. For this analysis, we removed data from all frequencies below 20 kHz and above 7.5 MHz since they often were impacted by contact artifact and inductive affects, respectively, and only added noise to the overall impedance spectrum, yielding EIM data for 34 frequencies. Data was first collected with electrical current flow passed parallel (longitudinal) to the muscle fibers and then, after rotating the electrode array 90°, perpendicular to the muscle fibers (transverse).

Histological analysis

After EIM measurements were completed, the left gastrocnemius was excised and fixed in 10% formalin. The tissue was then embedded in paraffin and sectioned into 10 µm slices. Sections were stained with collagen VI antibodies (Abcam #6588) to identify the cell membrane and 4', 6-diamidino-2-phenylindole (DAPI) to stain nuclei. Individual sections were viewed and photographed using a Zeiss Axioimager M1 Epifluorescence Microscope. Using Volocity® Software, (Perkin Elmer, Waltham, MA), myofiber area was identified using the automated algorithms that identify the fiber membranes and counts completed. On average, approximately 250 fibers were counted per muscle. Careful attention was paid to measure all cells that appeared to be myofibers, with the goal of measuring representative fields that accurately reflected the overall status of the muscle.

Data analysis

EIM data outputs included the resistance, reactance, and phase values at 34 frequencies in both the longitudinal and transverse directions (thus a total of approximately 204 outputs per animal). Prior to beginning the formal data analysis, we reviewed all EIM traces and removed any errant individual frequency points and performed a simple imputation technique in which the results for the frequency immediately below and above were averaged and substituted for the value obtained.

We followed an approach analogous to that previously used.³ Briefly, we first standardized the mean muscle fiber size and the impedance parameters to unit normal scale in order to remove the effect of the underlying unit of measurement. We then employed least absolute shrinkage and selection operator (LASSO) for assessing the entire multifrequency (resistance, reactance, and phase) components measures along the longitudinal and transverse directions without imposing any a-priori structure on them. The LASSO approach, which includes a penalty on the coefficients of the parameters, assumes that very

few components of the multi-frequency relate to the myofiber size. This assumption is essential as the number of components in the multifrequency set is much larger than the sample size in our study. The penalty defined as the absolute sum of the predictors' coefficients weighted by the tuning parameter, "shrinks" coefficients of the predictors that play very little role in explaining the response variability, and thereby "selects" the predictors that are strongly associated with the outcome. In our study, the penalty parameter in our model was selected using the leave-one-out cross-validation (LOOCV) approach. As a first step, a grid of values is specified for the penalty term in the above LOOCV procedure. We then split the sample into two sets: a validation and a training set. For a specific value of the penalty term, we build the model on the training set and then test its predictive performance (squared residual) on the observation that is in the validation set. As the name of LOOCV implies, we only include one random sample in the validation set while retaining the remaining observations in the training set. We repeat the above steps on the entire set of observations to obtain the average of Residual Square (which is an estimate of mean square error) for this particular value of the penalty term. The final penalty value is chosen to be one that provides a minimum mean square error on the entire grid of penalty. As a last step, the model is fit on the entire data for the final chosen value of penalty term to obtain the estimates of model parameters. The predictive models developed using the LASSO approach (with cross-validation) tend to have biased estimates of the parameters. However, these estimates also have relatively low variability in comparison to alternate models, and hence perform extremely well in the out-of-sample datasets ⁸

RESULTS

EIM prediction data and relationship to histology

Of the total number of mice originally studied (N=37), 8 animals were excluded in their entirety given that EIM from these mice data was markedly distorted by artifact across the entire frequency range in a single direction (i.e., transverse or longitudinal), as determined by visual inspection of reactance versus resistance curves. This included negative values at either high or low frequencies, values far outside the expected range (e.g., reactance values of greater than 200 ohms) or a non-physiologic impedance spectrum (e.g., a single major reactance peak at 1 MHz, rather than a peak at approximately 100 kHz and second upward trend above 1 MHz). In addition, 7 animals (i.e., 19% of data points (7 out of 37 = 18.9%)) had missing or egregious impedance data points at specific single frequencies. For these animals, data (14 values from a total of 5916 collected) was imputed as described above. This resulted in complete data sets from a total of 29 ALS animals from 8–18 weeks of age that were used in our analysis.

Figure 1 shows the basic longitudinal EIM data set obtained from female mice at 8, 12 and 16 weeks of age, including representative histological images, the impedance (i.e., reactance and resistance) versus frequency plots, as well as the Nyquist (reactance versus resistance) plots at each time point. With increasing age, and therefore with increasing disease severity, there is a gradual alteration in the impedance spectra, most easily observed in the Nyquist plots. Similar histological findings and impedance data were observed for the male ALS mice studied from 8–18 weeks (data not shown).

Our predictive model (based on the combined data sets from both female and male mice) using the LASSO strategy, and relying on EIM frequencies alone, gave an approximate RMSE of 14% (or 207 μm^2) for a mean fiber area of 1488 μm^2 . Figure 2A shows the actual LASSO regression comparing the predicted values (abscissa) to the actual values (ordinate) across these animals. A combination of longitudinal and transverse EIM data from only 10 frequency components was required to perform the prediction for either mean fiber size or coefficient of variation (CoV).

A major anticipated change of ALS-impacted muscle is the development of denervation atrophy—namely group atrophy with some fibers remaining of normal size and others being substantially reduced in size often in patchy fashion. Such a mixed size fiber population would increase the CoV (i.e., the standard deviation of fiber size/mean of fiber size) of the fiber size; the greater the variation in the fiber size, the greater the CoV. Indeed, a similar prediction model predicted the CoV. The results of this analysis are shown in Figure 2B with a RMSE of only 8.8%.

Supplementary table 1 provides the exact frequencies derived from the respective prediction models that were used to calculate the mean fiber area and the CoV.

DISCUSSION

We have demonstrated that by using non-invasive surface-based electrical impedance measurements (EIM) and a prediction model it is possible to achieve a reasonable estimate of the degree of myofiber size and variation in muscle impacted by ALS, including the degree of relative atrophy in the muscle and the distribution of myofiber sizes.

Previous work in ALS has evaluated EIM in comparison to standard electrophysiological parameters, including the motor unit number estimate (MUNE) and the compound motor action potential (CMAP) amplitude^{9,10}. Those studies identified a strong relationship between EIM and these parameters. In addition, we have evaluated the relationship between EIM and force output in ALS mice and again found a robust relationship¹¹. It is perhaps not unexpected, therefore, that we have identified a similar relationship between EIM and muscle fiber size here. The novelty of this work from those earlier studies is that in the present analysis we were seeking to identify a predictive model—in other words, we are utilizing raw impedance data alone to predict myofiber size rather than simply establishing a correlation between the EIM parameters and the electrophysiological/functional parameters. Also, we generated the predictive model using the entire frequency set and the predictive LASSO approach rather than basing our analysis on just a single frequency or subset of frequencies (as has been the case for most studies). The value of this approach is that anyone using the same methodologies as we have performed here can use the coefficients derived in this prediction equation to approximate myofiber size in their sample provided that the same electrode array is used and frequencies measured.

In some respects, myofiber atrophy in ALS is a secondary effect—it is, after all, merely a downstream result of motor neuron loss. Nevertheless, having a tool to non-invasively quantify myofiber size and distribution could serve a variety of purposes in ALS research.

First, it could provide a means for assessing the effect of therapies geared specifically to impacting muscle health, which remains very much a possibility either as a primary or secondary/adjuvant therapy.¹² In addition, it is possible that this data set could be used in conjunction with standard measures, such as CMAP and MUNE, to provide a broader sense of end organ health and effects of therapy in this disease.

More broadly, this study demonstrates that the technology and this approach have the potential to be used in other neuromuscular disorders with substantial fiber atrophy. One of our reasons specifically for choosing to study the ALS animals is that they tend to develop only fiber atrophy with little other significant intramuscular pathology. Similarly, in other conditions in which there is primary myofiber atrophy with little other accompanying interstitial change (e.g., disuse, sarcopenia, and corticosteroid use) this same approach can be utilized. However, it may also be possible to use this approach even when there are additional superimposed pathological features in the muscle tissue (e.g., connective tissue deposition or intracellular abnormalities), although this is yet to be determined and remains the goal of further study.

It is worth considering the mechanisms of fiber atrophy in ALS. Whereas some fibers may be normal and entirely unaffected and others are severely atrophied, likely related to complete denervation, the histopathological analysis revealed that in the ALS animals many fibers were actually smaller than normal but not severely atrophied. There are several possible reasons for this fact, including the presence of recently denervated fibers (i.e., those in the process of shrinking), recently reinnervated fibers (i.e., those in the process of growing), and reduced leg use producing secondary disuse atrophy.

Compared to our one other study that adopted an EIM-based predictive approach,³ there were several major differences here as alluded to in the introduction. First, in that study we used needle electrodes placed only one direction (longitudinally). The reason for that decision was mostly practical as the hind limbs in the early postnatal mice were too small to accommodate a surface electrode array or to perform transverse measurements. However, by doing so, we also removed one potential confounding factor: the skin and subcutaneous fat, which contribute to some of the artifact-related challenges described above. In addition, the skin and fat will impact the data set to at least some extent¹³ likely somewhat weakening the technique's predictive capability, especially if those tissues alter during the disease course along with the myofiber atrophy. Second, since the animals in that previous study were all healthy, they had a normal fiber size distribution; in other words the CoV in fiber size was identical across all post-natal ages examined. In the ALS animals studied here, there was considerable variation in the distribution of fiber size with some preserved fibers, some already quite atrophied, and others in between. This range in pathology is reflected in the distribution of the CoV that we observed. Accordingly, we would not expect the same frequencies to be identified as relevant predictors. Nevertheless, 4 of the 14 parameters in that study were used here (428 kHz reactance, 1027 kHz resistance and phase, and 5915 kHz phase).

There are several limitations to this study. Most importantly, we did not employ a completely separate test group, but rather used the approach of multiple sampling with

cross-validation from the same group. Analysis of a separate test group is challenging to achieve due to the large number of animals that would be required. Nevertheless we did perform a more basic k-fold cross validation approach¹⁴ (splitting the set into smaller groups and using one for training and one validation) and found that it gave us similar results. However, our leave-one-out cross validation approach is more appropriate for this small data set since it helps to ensure that the prediction model developed here does not over-fit the observed outcome. Second, we were forced to remove 22% (i.e., 8 out of 37) of animals from the original data set due to poor EIM data quality. Improved approaches for evaluating data quality at the time of collection are now underway such that artifacts impacting data can be avoided. One possibility would be to produce an automated algorithm that can help screen and remove such data prior to analysis; this is currently in development. Doing so would help reduce any bias that may occur when doing so by simple visual inspection, as we have done here. Finally, the critical EIM parameters identified in these prediction models, while being valid for the ALS mouse, could not be applied directly to humans, without actually developing a separate model that would once again require correlation of EIM values with histological data. Accordingly, this methodology would likely be best limited to pre-clinical research in which longitudinal assessments on animals are being performed and euthanasia is to be avoided.

In summary, we have shown that the surface based technique of EIM in conjunction with a prediction algorithm can effectively predict myofiber size non-invasively in an ALS mouse model. Further study of this approach in ALS research and in additional animal models of neuromuscular disease may provide additional insights into how this approach can be used most effectively. This will also include the use of dedicated EIM needle electrodes or combined EIM/EMG electrodes such that morphological and compositional data can be acquired along with standard electrophysiological measures.¹⁵ Our laboratory also plans to continue to this work by determining the capability of the EIM technology to assess other compositional alterations of the muscle tissue, including the presence of both extracellular and intracellular pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALS	amyotrophic lateral sclerosis
CMAP	compound motor action potential
EIM	electrical impedance myography
DAPI	4',6-diamidino-2-phenylindole

kHz	kilohertz
LASSO	Least absolute shrinkage and selection operator
LOOCV	Leave one out cross validation
MUNE	motor unit number estimate
RMSE	root mean square error
SOD1	superoxide dismutase 1

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Figure 1.

Compilation of representative histological images and averaged multifrequency EIM data from ALS mice with different degrees of muscle atrophy. A. Representative muscle histology from three female ALS mice euthanized at 8, 12, and 16 weeks of age, respectively (stained with antibodies to collagen VI (red, cell membranes) and DAPI (blue, nuclei)), bar = 20 μ m. B. Mean Longitudinal Reactance ($\pm$ standard error) versus frequency for separate cohorts of female mice euthanized at 8, 12, and 16 weeks of age (N=3–4 per time point); C. Mean Longitudinal Resistance ($\pm$ standard error) versus frequency for separate cohorts of female mice euthanized at 8, 12 and 16 weeks of age (N=3–4 per time point); D. Nyquist Plots: Average Longitudinal Reactance plotted versus Average Longitudinal Resistance at all frequencies for the three cohorts of female mice analyzed at 8, 12, and 16 weeks of age. Note changes in Longitudinal EIM spectral features (e.g., increased reactance and resistance at the low frequencies; alterations in the shape of the Nyquist plots) with advancing disease.

Figure 2. Observed versus predicted data. A. Mean fiber size; B. Coefficient of variation. Note the relatively linear relationship between these feature sets.