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Electrical impedance myography for monitoring motor neuron loss in the SOD1 G93A amyotrophic lateral sclerosis rat

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Abstract

Objective—Human studies have shown that electrical impedance myography (EIM), a technique based on the surface application of high-frequency, low-intensity electrical current to localized areas of muscle, is sensitive to muscle denervation. In this study, we examined the role of EIM as a potential biomarker for assessing ALS disease progression in the SOD1 transgenic rat by comparing it to motor unit number estimation (MUNE).

Methods—Multi-frequency EIM and MUNE were performed twice weekly in 16 rats from approximately 10 weeks of age onward. Four different EIM measures were evaluated, including the previously studied 50 kHz phase and three condensed multi-frequency parameters.

Results—The rate of deterioration in the multi-frequency phase data from 100-500 kHz had the strongest correlation to survival ($\rho = 0.79$, $p < 0.001$), surpassing that of MUNE ($\rho = 0.57$, $p = 0.020$). These two measures were also strongly correlated ($\rho = -0.94$, $p < 0.001$) to one another.

Conclusions—These findings support that EIM is an effective tool for assessing disease progression in the ALS rat.

Significance—Given its ease of application and ability to assess virtually any superficial muscle, it deserves further study as a biomarker in human ALS clinical therapeutic trials.

Keywords

amyotrophic lateral sclerosis; electromyography; motor unit; biomarker

Introduction

Amyotrophic lateral sclerosis (ALS) remains a devastating disease with minimal effective therapy (Qureshi et al., 2009). A major roadblock to the assessment of new treatments in ALS is the lack of effective, sensitive biomarkers for the survival (Cudkovic et al., 2010). A variety of radiological and serologic biomarkers have been studied (Turner et al., 2009). However, since the final common pathway of ALS is motor neuron loss and consequent muscle wasting, motor-specific markers would seem to offer a promising approach. Indeed, one technique, motor unit number estimation (MUNE), has been studied in ALS for more than three decades (Shefner and Gooch, 2003). The aim of MUNE is to approximate the

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number of motor neurons innervating a given muscle and is thus conceptually appealing (Dantes and McComas, 1991). A variety of methods for performing MUNE have been developed, including the incremental (McComas et al., 1971) and multipoint techniques (Brown, 1972). A combination of the two methods may offer better reproducibility and ease of application in clinical trials (Goyal et al., 2010; Shefner et al., 2011). However, regardless of specific technique, MUNE suffers from several limitations, including its requiring considerable training and ongoing decision-making as data is collected, its ability to evaluate only a very limited set of distal muscles, and only fair repeatability early in the disease course (Felice, 1995).

Electrical impedance myography (EIM) is another electrophysiological technique that holds promise for assessing ALS progression (Rutkove, 2009; Rutkove et al., 2007). Unlike standard electrophysiological techniques, EIM is not directly dependent upon the inherent electrical potential of muscle or nerve tissue. Rather, in EIM, high-frequency alternating electrical current is applied to localized areas of muscle via surface electrodes and the consequent surface voltage patterns analyzed (Rutkove, 2009). Changes in muscle mass, composition, and internal structure that occur with muscle denervation and reinnervation impact the impedance signature, altering the surface impedance measurements across the frequency spectrum (Esper et al., 2006). EIM has its foundation in the realm of bioelectrical impedance, but unlike standard bioimpedance approaches, the measurements are performed over discrete areas of muscle with detailed attention to electrode orientation and spacing. Previously, we showed that the technique has excellent sensitivity to human ALS progression (Rutkove et al., 2007).

Although it is straightforward to perform studies of both EIM and MUNE in humans, the ALS G93A rat model presents a unique opportunity for biomarker assessment. This is true because measurements can be performed at very frequent intervals and the data correlated to motor neuron number in the spinal cord. In addition, thanks to an inadvertent alteration in transgene copy number, currently available animals progress at considerably varying rates (Herbik et al., 2006). Although such variability is undesirable for therapeutic studies, it is actually quite advantageous in a biomarker study where a wide dynamic range of disease progression is needed. Thus, in this study we evaluate the capability of EIM and MUNE to serve as biomarkers of motor neuron loss and the relationship of those values to survival in the SOD1 G93A ALS rat.

Materials and Methods

Animals

Sixteen male SOD1 G93A rats were obtained from Taconic Laboratories (Germantown, NY), at 8-12 weeks of age. Animals were allowed to acclimate at least 48 hours prior to use in any studies, housed singly, and fed a regular diet *ad libitum*. All studies were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. Animals were studied weekly initially and then twice weekly as they began to clinically deteriorate, based on their beginning to lose weight (Weydt et al., 2003). No control animals were studied here as these have already been demonstrated to show stable EIM data from approximately 14 weeks of age onward (Ahad and Rutkove, 2009).

Animal preparation

Prior to the studies being performed, animals had the fur overlying the left gastrocnemius muscle removed first with clippers followed by application of a depilatory agent. To ensure similar positioning of electrodes for both MUNE and EIM from week to week, a pinpoint tattoo was applied over the left gastrocnemius as a reference point. During each study

session, animals were weighed, followed by MUNE and EIM. During the latter two procedures, the animals were maintained under isoflurane anesthesia delivered at 2.5 L/minute via a nosecone. Animals were placed in the prone position with the left limb affixed with adhesive tape and spread at an approximately 45 degree angle to the spine (Ahad and Rutkove, 2009).

Electrical Impedance Myography

EIM measurements were performed on the rat as previously described and as shown in Figure 1A, a method that has shown high reproducibility (Ahad and Rutkove, 2009). The impedance measuring system consisted of a multi-frequency lock-in amplifier (Model 7280, Signal Recovery, Oak Ridge, TN) coupled with a very low capacitance active probe (Model 1103 of Tektronix, Beaverton, OR) providing data from 0.5-1000 kHz (Esper et al., 2006). The two parameters obtained from this system are resistance (R) and reactance (X) from which the third parameter, phase (θ) is calculated via the relationship $\theta = \arctan(X/R)$. From the obtained spectrum, the 50 kHz data was extracted as were several “collapsed” parameters that represented the characteristics of a portion of the spectrum in single variables, including the reactance-slope from 100-500 kHz, the phase-slope from 100-500 kHz, and the resistance log-slope from 10-500 kHz (Rutkove et al., 2010). Briefly, the reactance- and phase-slope were obtained by first performing a least-squares fitting of all the data obtained from 100-500 kHz and then taking the slope of the resulting line; the resistance log-slope was obtained similarly from the 10-500 kHz, but first applying a log-transformation on the resistance and frequency values, which has the effect of making the resistance-frequency relationship nearly linear. For all three of these collapsed variables, increasing value indicates progressing disease.

Motor Unit Number Estimation

MUNE was performed using a TECA Synergy T2 EMG Monitoring System (Viasys, Madison, WI) on the left hind leg in a position identical to EIM, stimulating the sciatic nerve at the sciatic notch (Neuroline #746 12-100/25 needle electrodes, Ambu Corp, Denmark) and recording via disposable ring electrodes (Product # 019-435500, Faith Medical Inc., Steedman, MO) around the entire distal leg (Figure 1B) (Shefner et al., 2002). A ground electrode was also placed on the right hind paw. All MUNE was performed by a single individual (LW) using the standard incremental method after careful training in the technique by Dr. Rutkove (McComas et al., 1971), including repeated practice on several normal animals. An initial compound motor action potential (CMAP) was obtained using this set-up. Stimulus intensity was then decreased to zero and gradually increased until the first single motor unit potential was identified, required to have a minimum amplitude of 25 μ V and to be consistently present. Stimulus intensity was then gradually increased until a consistent step in amplitude was identified of at least 25 μ V. The procedure was then repeated up to 10 times attempting to always find the smallest step. Each of these values was subtracted from one another to obtain the amplitude of individual steps and the average of the value taken as the mean single motor unit potential amplitude. This value was then divided into the initial CMAP obtained.

Timing of Sacrifice

The animals were sacrificed when they could no longer effectively feed themselves (when either both the fore or hind limbs were completely paralyzed). After a final set of EIM and MUNE measurements, the rat was euthanized via exsanguination under anesthesia and perfused with 0.4% paraformaldehyde.

Spinal cord

The lower lumbar spinal cord was extracted following standard protocols and cut at 10-micron thick sections. H&E staining was performed and the motor neurons in the anterior horn were counted across 20 sections spaced 100 microns apart (2 mm) corresponding to the side and approximately the L5-S1 levels based on the location of the exiting nerve roots. The density was calculated by the taking the average number of motor neurons counted across the 20 sections and dividing by the length of spinal cord from which they were taken (2mm).

Statistics

For all correlation analyses, Spearman rank correlations were performed. Two-group comparisons were made using the Mann-Whitney test. For all statistical tests, significance was accepted at $p < 0.05$, two-tailed. Analyses were performed using MATLAB (Mathworks, Inc, Natick, MA)

Results

Animals and weight loss

As anticipated, the animals' life spans varied considerably (Figure 2), with survival ranging from approximately 155 days to 225 days. The animals' weights tracked accordingly. Despite considerable variation in survival, all animals reached peak weight at approximately 130 days, and thus this value was taken as the date of clinical disease onset, as has been adopted by others (Weydt et al., 2003); this was also the point at which we began twice-weekly measurements.

Prototypical changes in EIM parameters over time and derivation of collapsed parameters

Figure 3 shows the prototypical changes in the multi-frequency spectrum taken from a single animal over time. As can be seen, each EIM spectrum becomes greatly altered as the disease progresses. The goal of the multi-frequency collapsed parameters is to capture the alteration in the frequency spectrum with time, rather than relying on a single frequency value, which is more prone to noise and electrode positioning variation (Rutkove et al., 2010).

Comparison of EIM and MUNE parameters over time

Figure 4 shows the alteration in each of the four EIM parameters, CMAP, and MUNE over time for all the animals. As can be seen, all parameters show similar trends (although, as anticipated based on our previous study (Rutkove et al., 2010), the multi-frequency EIM parameters move in the opposite direction of MUNE, CMAP, and 50 kHz phase and increase over time).

Assessment of the rate of disease progression and correlations to death

In order to determine the rate of progression as measured by a given parameter over time, we obtained a slope of decline from a linear fit for all the twice-weekly data points obtained from each animal starting at 130 days, the approximate time of maximal weight, and ending at their death, essentially mimicking the type of data that is required when employing the standard mixed model approach used in clinical trials work. Since the purpose of a biomarker in ALS is to serve as surrogate measure of survival, a relatively strong relationship between the rate of alteration in that marker and the length of survival is desired; thus, a correlation analysis between that rate of deterioration and survival length was performed (Table 1). Of all measures, the phase-slope provided by the far the strongest correlation between rate of disease progression and death, with $\rho = 0.79$, $p < 0.001$; MUNE, in contrast, had a correlation of 0.57, $p = 0.02$ (Figure 5).

Similarly, we compared the coefficients of variation (standard deviation in the rate of deterioration divided by the mean rate of deterioration) for each of the parameters and these are also included in Table 1. The coefficient of variation provides a simple index for assessing a biomarker's ability to serve as an effective outcome measure in a clinical trial. Although the coefficients obtained here cannot be compared in a meaningful way to human data (in which, for example, the ALSFRS-R has a value of about 1.0) (Cudkovicz et al., 2006), their relative strength to one another within this study is still meaningful. As can be seen, all are fairly close in value and the phase-slope value of 0.52 fares well compared to the other measures, including MUNE with a value of 0.54. Thus, for simplification and given the phase-slope's strong correlation to survival, in the following analyses we focus only on two parameters: phase-slope and MUNE.

Next, we compared the group of shortest-lived animals to the group that lived longest. This data can be effectively presented as a Kaplan-Meier analysis by separating the 2 groups by rate of progression of the two parameters (Figure 6). For both of these approaches, EIM phase-slope fared better than MUNE as an index of separation. For MUNE, mean rate of change for the fastest progressing animals was 3.63 motor units per day (range 2.33-5.24) and for the slowest progressing animals 1.47 motor units per day (range 1.23-1.89), which corresponded to a non-significant difference in median survival ($p = 0.103$). For EIM phase-slope, mean rate of change for the fastest progressing animals was $0.19^\circ/\text{MHz}$ per day (range 0.085 to 0.27) and for the slowest progressing animals was $0.45^\circ/\text{MHz}$ per day (range 0.29 to 0.62), which corresponded to a significant difference in median survival ($p = 0.040$).

Correlations between EIM and MUNE

Figure 7 shows a scatter plot of the average weekly MUNE across the cohort of animals to the EIM data from the animals from each of the same measurement sessions, showing a strong correlation between the two methods with $\rho = 0.94$, $p < 0.001$.

Motor neuron counts correlations

The average motor neuron density was very low, ranging from 2.8 to 17.9 motor neurons per 1 mm length of spinal cord across the sixteen animals. No significant correlation to motor neuron density in the lumbar cord was identified in any of the EIM or MUNE parameters averaged across the last two weeks of measurements.

Discussion

There were three main purposes to this work. First, we were seeking to understand EIM's potential role as a marker of disease progression and prognosticator of survival in ALS in comparison to MUNE. Second, we wished to determine which of several EIM markers appeared most promising, anticipating that a multi-frequency measure would outperform the 50 kHz phase data we have used for most of our earlier studies. In fact, recent work in spinal muscular atrophy has also supported that multi-frequency measures outperform single frequency ones (unpublished results). Finally, we wished to understand the relationship between EIM and MUNE.

As Figure 3 suggests, both MUNE- and EIM-based measures show evidence of clinical disease onset by about 130 days on average for the group of animals, at about the same time the animals reached their maximal weight. There is no gold standard for identifying disease onset and, in fact, other studies both in the ALS mouse and rat have shown that a variety of subclinical abnormalities can actually be identified at very young ages (Chen et al., 2010; Graber et al., 2010). Thus, neither MUNE nor EIM, in this analysis, appears especially useful in identifying disease onset.

Perhaps more importantly, both EIM and MUNE correlate well with survival and have similarly low coefficients of variation for this cohort of animals. Of the several EIM parameters, EIM phase-slope appeared to be the most robust. Not only did it fare better compared to the other EIM parameters in terms of its correlation to survival, but it also fared better compared to MUNE. Accordingly, it was also better at discriminating the fast- from the slow-progressing ALS animals. EIM phase-slope was also very strongly correlated to MUNE, with a $\rho = 0.94$, $p < 0.001$. In general, the EIM parameters also showed more linear changes with disease progression than did MUNE (again as shown in Figure 3); for clinical trials purposes, having a parameter change in a linear fashion is also highly desirable.

There are several advantages to performing this biomarker study in animals over human subjects. First, we are able to obtain frequent, consistently spaced measurements, on all the animals thus making for a far more careful and precise experiment than is typically possible with biomarker studies in human subjects. Second, we could avoid the presence of superimposed illnesses and age variation that also impacts most clinical studies. Third, we were able to measure the animals before they had developed any clinical signs of the disease and could thus evaluate sensitivity to onset. Finally, although negative, we were also able to assess the relationship between these electrophysiological measures and motor neuron number in the spinal cord.

Regardless of these advantages, there remain several limitations to this study. First, we only evaluated one muscle in one limb, seeking this single measure to serve as a proxy for disease activity in the entire organism. In point of fact, such single-muscle evaluation is standard for MUNE as used in clinical trials since the procedure is time-consuming and it can only be easily performed on muscles easily accessible to peripheral nerve stimulation. EIM when applied in humans, in contrast, can be performed on any of a number of muscles, given its speed of application and lack of reliance on nerve stimulation. Thus, in a clinical trial it may be preferable to choose a group of muscles to follow over time or to perhaps follow just one muscle that is expected to deteriorate most rapidly during the study period (Rutkove et al., 2007). Other limitations of this work include the fact that we included only male rats and had no functional measurements (e.g., rotarod). Moreover, our pathological comparisons failed to reveal a positive association between either MUNE or EIM with spinal motor neuron number. This last finding is perhaps not surprising given that we sacrificed animals only when they had reached end-stage, a point at which each animal had a very low motor neuron count. To do such a study properly would require sacrificing animals at different stages of the disease process, which was not possible given our experimental design. Finally, we utilized the more basic form of incremental MUNE and not the newer promising modified incremental technique (Shefner et al., 2011).

Given that EIM evaluates structural and compositional alterations in muscle caused by denervation and MUNE assesses motor neuron loss, it may appear unexpected that EIM should show a considerably stronger correlation with survival than MUNE while still achieving a similar coefficient of variation. One potential explanation may lie in EIM's reproducibility, which has previously shown to be quite high in both human and animal studies (Ahad and Rutkove, 2009; Rutkove et al., 2006). MUNE, on the other hand, generally has lower reproducibility especially early in disease course (Felice, 1995; Shefner, 2001). Inconsistent values could result in more variable slopes of decline for MUNE due to inaccurate least-squares fitting of the data and ultimately a lower correlation with survival. The less linear decline, as suggested by Figure 4, could also contribute to this lower correlation with survival.

Thus, using the SOD1 G93A rat model, we have shown that EIM can serve as an effective marker of disease progression in ALS. Although it fares comparably to MUNE in many of

these analyses, EIM has a number of important advantages including that it is completely non-invasive and painless, that it requires minimal training and is very fast to apply (with a complete data set obtainable within seconds), and that it is possible to assess many different muscles rather than being restricted to a few distal limb muscles. Indeed, EIM can be used to evaluate the tongue and thoracic musculature, offering the possibility of assessing deterioration in regions not previously accessible to such measurements. Additional human data collection and device development for improved application of the technique is now underway (Ogunnika et al., 2010).

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Highlights

- Electrical impedance myography has a strong correlation to motor unit number estimation in the ALS rat
- The rate of decline in Electrical impedance myography correlates strongly to life expectancy of the animals.
- Electrical impedance myography progresses linearly, consistent with human data collected thus far, and supporting its potential ease of application in ALS clinical trials.

Figure 1.
(A) EIM and (B) MUNE, performed on the rat gastrocnemius muscle.

Figure 2. Histogram showing survival times for the cohort of animals; weight of individual animals from 85 days until death (fastest progressing animals in black, slowest progressing animals in gray).

Figure 3.

Typical changes in EIM spectral data in a single animal over time. Multi-frequency data for resistance, reactance, and phase in an individual animal at 124, 162, and 187 days of age, just before the animal's death.

Figure 4.

EIM and MUNE parameters plotted from 100-200 days of age. The MUNE parameters drop quickly at about 145 days and then level off, whereas the EIM parameters show a more linear alteration over time.

Figure 5. Scatter-plot comparing rate of progression from 130 days until sacrifice with MUNE and EIM phase-slope.

Figure 6. Kaplan-Meier curves for MUNE and EIM phase-slope rate of progression. Animals with fastest rate of deterioration based on each parameter in black, animals with the slowest rate of deterioration in gray.

Figure 7. Relationship between MUNE and EIM phase-slope from time of 130 days (approximate age of onset) to 185 days (last date of measurement with more than half the cohort of animals alive). Data for each week of measurement is averaged over the entire group of animals.

Table 1
Correlation between electrophysiologic data and individual animal survival

	Correlation [*] between rate of deterioration and lifespan	p-value	Coefficient of variation in rate of decline
CMAP	0.57	0.02	0.69
MUNE	0.57	0.02	0.53
50 kHz θ	0.35	0.18	0.47
R log-slope	0.35	0.25	0.43
X-slope	0.61	0.013	0.63
θ-slope	0.79	$p < 0.001$	0.52

^{*} Spearman correlation