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Neuromuscular fatigue detection by mechanomyography in people with complete spinal cord injury

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Abstract

Purpose Functional electrical stimulation (FES) is a method of activating paralyzed muscles. During FES application, fast muscle fatigue can occur (the inability of stimulated muscles to generate force). Therefore, it is beneficial to estimate the muscle fatigue for FES closed-loop control for walking to prevent unexpected muscle collapse and adapt the FES strategy in real time. Mechanomyography (MMG) is a noninvasive technique for registering myofiber vibrations, representing an ideal candidate for the provision of feedback. The hypothesis was that MMG signals could effectively detect muscle fatigue and, thus, provide feedback.

Methods We tested this hypothesis by analyzing the wavelet transform of signals from an MMG sensor positioned over the *rectus femoris* muscle during electrically evoked contractions in subjects with spinal cord injury (SCI). The signals were collected from a total of 24 lower limb muscles. We investigated both legs of 15 participants with spinal cord injury (male, YOA = 27.13 ± 5.05 , M = 75.8 ± 10.35 kg, and H = 1.78 ± 0.07 m, American Spinal Injury Impairment Scale (AIS) A and B). All MMG signals were analyzed in 12 frequency bands from 5 to 53 Hz.

Results We found different trends in the magnitudes in different frequency bands. The magnitude decreased in 13, 16, 20, 25, and 35 Hz bands in correlation with fatigue. The greatest statistical difference was found at 20 Hz and 25 Hz.

Conclusion This result suggests that processed MMG signals indicate muscle fatigue and can, thus, be used as the feedback in FES systems.

Keywords Electrical stimulation · Evoked potentials · Lower limb · Muscle contraction · Vibration · Wavelet

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Abbreviations

FES Functional electrical stimulation

SCI Spinal cord injury
MMG Mechanomyography
EMG Electromyography

MEMS Microelectromechanical systems

AIS ASIA impairment scale

ASIA American Spinal Injury Association

MESE Maximum electrically stimulated extension

Introduction

Functional electrical stimulation (FES) has been used in rehabilitation for restoration of the muscle function of patients with spinal cord injury (SCI) (Popovic and Sinkjær 2010). However, its application for prolonged stimulation is limited due to fast occurring muscular fatigue. Several methods can



be used to slow down the fatigue (Deley et al. 2015). Variations in stimulation frequency, as well as the use of distributed and asynchronous stimulation, significantly increase the effective duration of sustained electrical stimulation (Malešević et al. 2010; Popović et al. 2013).

FES has been used to promote improvements in spinal excitability (Kim et al. 2015a, 2015b) and in whole-body composition, as described in Khalil (2015). When FES is applied on fatigued muscles, the resulting force is not adequate for desired motor function, and there is an increased delay in force generation (do Couto and de Sousa Oliveira 2016). Therefore, detecting muscle fatigue in real time would be useful for managing the FES strategy.

The direct method to assess fatigue is to measure the force and torque outputs during FES application to SCI subjects. However, the apparatus required for these measurements is bulky and non-convenient for non-isometric real-time measurements (Cè et al. 2015; Scheeren et al. 2018). Attempts have been made to use wearable neuromuscular fatigue detection systems based on electromyography (EMG) measurements (Bichler 2000). However, the obtained signals are distorted in the presence of FES and challenging to interpret, especially in cases of high-frequency stimulation (i.e., Russian stimulation) or variable frequency stimulation. The alternative is the assessment of myofiber displacement of skeletal muscle tissue by mechanomyography (MMG), which is immune to electrical interference from FES (Scheeren 2018). Cè et al. (2015) reviewed 111 papers related to MMG and found 20 of them focusing on neuromuscular fatigue in able-bodied subjects.

MMG is a technique that uses sensors on the skin surface to record neuromuscular vibrations during muscle contractions. MMG can be used to quantify the force generated in the muscle (Polato et al. 2008; Vaz and Herzog 1999). Typically, an MMG signal is recorded with an accelerometer that detects the vibration of myofibrils. Depending on the purpose and individual characteristics of the subject, there are several other types of sensors for detecting muscle vibrations (Krueger 2014).

Recent information indicated a reduction in the vibration of fast type II muscle fibers (high frequency) during neuromuscular fatigue. A clear indication of muscle fatigue is a noticeable shift in MMG signal spectral content towards lower frequencies. Bichler (2000) investigated single motor unit mechanical behavior in rats and found that the strongest type II muscle fibers (fast fatigable) generated the highest amplitude MMG signal. In paralyzed subjects, FES can mainly activate type II fibers. Zainah et al. (2017) identified different levels of muscle contraction in SCI subjects using an MMG sensor to measure torque during FES. Therefore, one can expect that fatigue would influence the decrease in the frequency spectrum component of the MMG signal.

In our previous study, FES caused muscle contraction, and MMG signals were acquired and processed in the time domain

(Krueger 2014). MMG magnitudes decreased when the *rectus femoris* muscle was fatigued in people with paretic lower limb SCI. The present paper intends to contribute to better understanding of the neuromuscular fatigue process in complete SCI subjects, aiming to promote new FES-MMG strategies in artificial motor control. The specific goal of this paper is to characterize *rectus femoris* muscle fiber vibrations, using wavelet transform, during electrically evoked contractions in subjects with complete SCI. MMG could help to predict muscle fatigue and aid in the development of optimal and personalized FES protocols. The hypotheses are that all frequency band magnitudes decrease with neuromuscular fatigue, and there is a more significant shift of frequency content towards lower values compared with the results from healthy subjects.

Methods

Subjects

We recruited 15 volunteers with SCI for this study.

Inclusion criteria: no voluntary contraction of the quadriceps muscle, patients classified as AIS A or B at levels between C5 and T10 on the ASIA scale

Exclusion criteria: cancer in the stimulated area of the lower limb; exposure to X-ray examination in the previous 2 weeks (aversion); epidural implant in the spinal cord, osteomuscular disorders, cognitive impairment, or intolerance to the sensation evoked by FES

None of the subjects had previously been involved in FES protocols.

The basic demography of the subjects is presented in Table 1. The SCI lesions ranged from C5 to T10, excluding participants with *cauda equina* syndrome (lesion at lumbar level). We used the American Spinal Cord Injury Association Impairment Scale (Schmidt-Read et al. 2011).

Figure 1 shows the protocol flowchart. Both limbs of all subjects were evaluated where possible. Six (right or left) limbs were excluded from the study due to the presence of metal implants (surgical pin), knee joint injuries, or sensation intolerance to FES in subjects with incomplete SCI (AIS B). Therefore, we evaluated only one limb (either right or left) in six subjects and both legs in nine subjects. During the measurement sessions, the temperature and relative humidity in the environment were 31.4 ± 2.28 °C and $43.4 \pm 10\%$, respectively. The skinfold thickness was measured at the MMG sensor site using a PrimeMed® adipometer.

Parameters of electrical stimulation

The electrical stimulator generated monophasic rectangular voltage-controlled pulses. The device was custom made and evaluated by a Biomedical Engineer respecting



 Table 1
 Participant

 characterization

Part.	Age (years)	Weight (kg)	Height (m)	Spinal cord injury		
				Level	Time of lesion (years)	Etiology
1	34	60	1.75	T7	1.33	Car accident
2	25	62	1.73	T7	0.75	Violence
3	25	65	1.89	T6-7	0.42	Violence
4	26	80	1.82	C7	1.00	Diving
5	24	86	1.74	T6-7	2.00	Car accident
6	19	70	1.86	T7	2.00	Violence
7	27	90	1.70	T6	2.00	Car accident
8	21	87	1.78	T7	1.58	Car accident
9	34	75	1.70	T5-6	2.00	Car accident
10	33	80	1.81	C5	2.00	Car accident
11	29	80	1.83	T10-11	2.00	Violence
12	22	60	1.69	T7-10	3.00	Car accident
13	24	77	1.89	C5	1.67	Car accident
14	29	76	1.79	T3-5	17.0	Violence
15	35	89	1.73	T10	2.33	Fall

Modified from (Cè et al. 2015)

the IEC 60601-2-10 standard (Morales 2003). The following parameters were used: The FES pulse frequency (carrier) was 1 kHz with a 20% duty cycle (i.e., 200 μ s

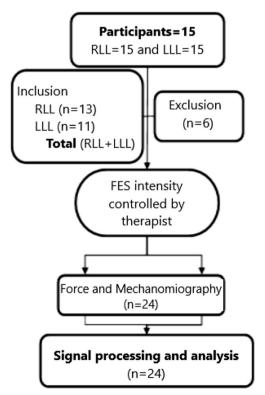


Fig. 1 Protocol flowchart. RLL, right leg limb; LLL, left leg limb. Six (right or left) limbs were excluded from the study. The therapist set the FES intensity and measured force and MMG signals. Collected signals were processed offline and analyzed

on and 800 μs off) to minimize the capacitive component of the skin-electrode impedance. The burst (modulating) FES frequency was set at 70 Hz with a 20% duty cycle (i.e., 2 ms on and 11 ms off) to speed up muscular fatigue. Two self-adhesive electrodes were positioned on the thigh: the anode (5 cm \times 9 cm) over the knee region and the cathode (5 cm \times 5 cm) over the femoral triangle to activate the quadriceps muscle via the femoral nerve.

Sensors

The MMG sensor consisted of a commercial Freescale MMA7260Q MEMS triaxial accelerometer that was hardwired to provide 800 mV/G sensitivity (G is gravitational acceleration), based on the component specification. The transducer was welded on a printed circuit board (13 mm × 18 mm). The mass of the whole assembly was only m =0.94 g. The output of the sensor was connected to the noninverting amplifier with the gain of A = 2.2. An Alfa Instrumentos® (Brazil) S-shape, aluminum load cell (50 kgf \approx 500 N) measured the force generated by the stimulation. The load cell was fixed to the base of the chair on one side and the subject's leg on the other side with Velcro strips. Both connections were rigid. Force measurement was performed in the isometric condition. To determine the fatigue epochs, the force signal was normalized to the percentile of the maximum force. Absolute muscle force (kgf) was determined to allow the comparison of the results between subjects.



The MMG sensor (Fig. 2) was placed on the skin over the *rectus femoris* (RF) muscle belly using 3 M® double-sided adhesive tape. The sensor placement was equidistant to the anterior superior iliac spine and the base of the *patella* bone. Figure 2 also shows the sensor axes orientation with signals X, Y, and Z representing the vibrations in the transversal, longitudinal, and perpendicular directions, respectively. We used a Velcro belt to stabilize the trunk.

Protocol

Subjects were seated on the chair with hip and knee flexed at 70°. The skin at the sensor site was shaved and cleaned. After the measurement of anthropometrical parameters, the subjects were warmed-up by passive knee mobilization. FES electrodes were placed on the lower limb, and then at least a 10-min rest interval elapsed to allow skin-electrode impedance stabilization (Reilly 1992).

Digital signal acquisition

A LabVIEWTM development platform was used for the acquisition of MMG signals. The acquisition system consisted of an NI-USB 6221 National InstrumentsTM board running at a sampling rate of 1 kHz.

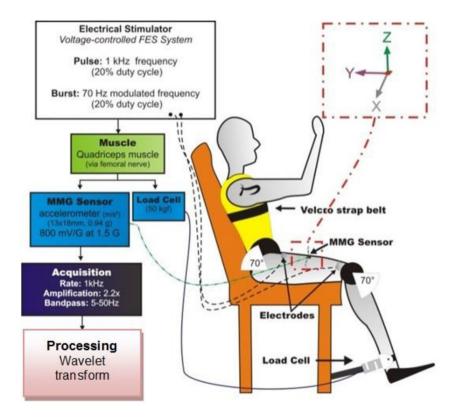
Fig. 2 Instrumentation layout and experimental setup. Modified from (Cè et al. 2015)

Determination of maximum electrically stimulated extension (MESE) voltage

Just before the load cell placement, the quadriceps muscle was electrically stimulated to extend the knee. When the knee reached full extension, the exact FES amplitude was registered as the maximum electrically stimulated extension voltage. This procedure was previously used in our experiments and named as maximum safety force (Rinaldin et al. 2020). After the load cell rigid attachment, the same FES intensity (MESE) was applied again, and the output voltage of the load cell was assumed to be the reference, $F_{100\%}$. Thus, the load cell output voltage was considered force normalized to the reference force and expressed as percentage of $F_{100\%}$. The 10-min resting interval was instigated to avoid interference in the fatigue protocol.

Fatigue evaluation

The isometric muscle contraction protocol was designed to cause muscle fatigue as fast as possible. Therefore, FES intensity was gradually increased to keep the load cell signal close to $F_{100\%}$ for as long as possible, but without spreading the stimulation to the antagonistic muscles. Figure 3 illustrates the normalized force. The points of time (PoT) of interest were determined using the following rationale: PoT I (non-fatigued muscle), FES intensity was manually adjusted to make the





load cell signal oscillate at approximately $F_{100\%}$; PoT II (prefatigue) when the controlled FES could not maintain the force at $F_{100\%}$; PoT III (partially fatigued muscle) when the output signal decreased to $F_{65\%}$; and PoT IV (fully fatigued muscle) when the force signal decreased to $F_{30\%}$.

Signal processing

MMG signals were processed in MatLab® (v. R2008a) and filtered using a 3rd order 5–50 Hz bandpass Butterworth filter. The x-axis, transverse to the anatomical position, was considered the best MMG axis for assessing the changes related to muscular fatigue in paraplegic subjects (Krueger et al. 2014).

Noise cancelation and baseline definition

The mean value of a signal in a 1-s window without muscle contraction was calculated and defined as noise magnitude. This value was used to compensate all signals. Negative values were set to 0 V to establish a baseline. Force was calibrated to initiate at 0 kgf.

Cauchy wavelet transform

The MMG signal was processed using Cauchy wavelet (CaW) transform (von Tscharner 2000). CaW is a normalization method for equality to be valid on all scales in the wavelet domain. Its value results from the multiplication of the CaW of the frequency bands and the intensity normalization factor (Protázio and Remacre 2002). The decomposition in frequency bands was defined by Eq. 1, being f as the frequency, f_c as the center frequency, f_c as the scale factor, f_c as the center frequency level, and f_c and f_c as the constants 1.45 and 1.959, respectively.

$$f_c(s,j) = \frac{1}{s} (j+q)^r$$
 (1)

Fig. 3 Fatigue protocol from representative participant. Red line, force; blue rectangle, instant I (non-fatigued muscle, $F_{100\%}$); green rectangle, instant II (prefatigued, $F_{100\%}$); yellow rectangle, instant III (partially fatigued, $F_{65\%}$); red rectangle, instant IV (fatigued muscle, $F_{30\%}$). Modified from (Cè et al. 2015)

where s can have values from 1 to 4 and j can have values from 0 to 20. Twelve out of 21 frequency bands were selected (between 5 and 53 Hz), as shown in Fig. 4. We selected only 12 frequencies to reduce the complexity of data presentation.

Due to the non-orthogonality associated with CaW (von Tscharner 2000), a normalization method was employed to preserve signal magnitudes in the wavelet domain. $C_{k, j}$ is an intensity factor obtained by multiplying the CaW of the frequency bands by the intensity normalization factor, $c_{k,j}$ (Eq. (2)).

$$c_{k,j} = \frac{1}{\sqrt{\left[\left(\psi(f_k, f_{c_{j-1}})^2\right) + \left(\psi(f_k, f_{c_j})^2\right) + \left(\psi(f_k, f_{c_{j+1}})^2\right)\right]}}$$
(2)

where k is the index that indicates a frequency interval $\left[0, \frac{F_s}{2}\right]$, F_s is the sampling rate (1 kHz), j is center frequency level, f is the frequency, ψ is the wavelet function, and c_j is the intensity factor. Figure 4 displays the distribution of each frequency band (f_c) .

Analysis window length

Epochs of 1-s duration were extracted from a discrete signal with a 1-kHz sampling rate (Krueger et al. 2013). In Eq. 3, CaW is the mean value of data processed with wavelet transform (CaW), fc is the central frequency of each frequency band, and PoT is the protocol instant (I-IV).

$$CaW_{fc,PoT} = \frac{1}{n} \sum_{n=1}^{1000} |CaW_{fc,PoT}|$$
 (3)

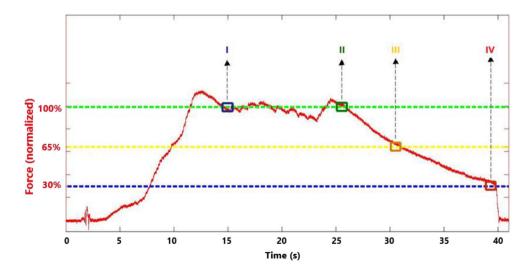
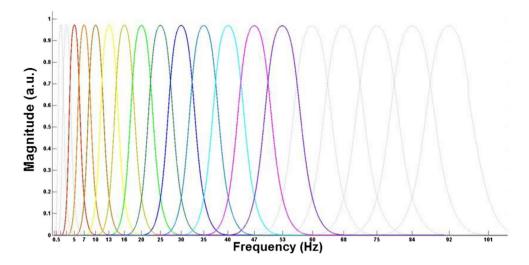




Fig. 4 Frequency bands obtained from Cauchy (modified) wavelet transform. Only the color frequency bands were computed



Normalization process

Data were normalized by the maximum of the twelve frequency bands in the non-fatigued muscle (PoT I), thus generating a magnitude scale in arbitrary units (a.u.), according to Eq. 4.

$$CaW_{Normfc,ins} = \frac{CaW_{fc,ins}}{max\left(CaW_{fc_1(\dots)fc_{12},I}\right)} \tag{4}$$

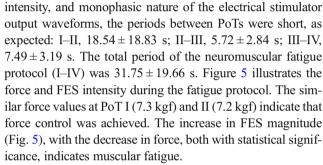
where CaW Norm is the normalized data.

Statistical analysis

In addition to the descriptive analysis, statistical tests were applied to find any significant differences between the chosen PoTs (I, non-fatigued muscle; II, pre-fatigued; III, partially fatigued, and IV, fully fatigued muscle) for the normalized magnitude of each frequency band. The statistical analysis was performed in MatLab ® (MathWorks, Inc. v. R2008a). A normality test on the features-to-be-evaluated yielded a negative result. The Friedman test was applied to the data (p value ≤ 0.05) to evaluate possible differences between the PoTs in the protocol. Considering axes with statistical significance, the Wilcoxon signed rank test was performed for post hoc analysis. The p value was chosen according to the Bonferroni adjustment for multiple comparisons; in this sense, differences were considered significant for $p \le$ 0.0125. The Spearman (ρ) correlation coefficient compared chronological (injury date) and pathological factors (injury level) with the force output and FES intensity required to create MESE (PoT I, non-fatigued muscle).

Results

Considering the neuromuscular condition of paraplegic individuals (i.e., untrained and atrophied muscles), high FES



The Spearman (ρ) correlation coefficients determined in this study were not significant between SCI time of lesion and force output, SCI time of lesion and MESE voltage, and SCI level and force output. Thus, these results were not affected by sample characterization.

Figures 6 and 7 show the CaW frequency band changes during the neuromuscular fatigue protocol, with a noticeable and significant amplitude reduction at 25 Hz.

Discussion

During the process of FES-induced neuromuscular fatigue, intrinsic neuromuscular alterations occur in people with SCI. Unlike previous studies that only expressed this relation through the force response, in this paper, we measured intrinsic muscular changes by MMG. Considering there is no influence of the electrical stimulation frequency on the MMG signal (Scheeren 2018), the significant contribution of this research is to point out the specific frequency band, obtained from CaW transform, in which fundamental changes in the vibration pattern of muscle fibers occur during the FES fatigue induction protocol.

According to Bigland-Ritchie and Woods (1984), any reduction in the force output indicates neuromuscular fatigue. Considering the absolute values of force presented in Fig. 5, a



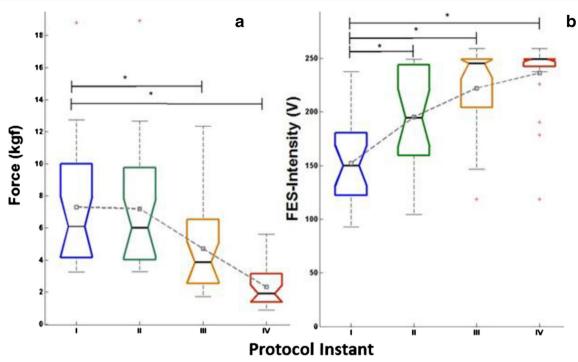


Fig. 5 Boxplot from force (**a**) and FES intensity (**b**) during fatigue protocol. Instant: I (non-fatigued muscle, $F_{100\%}$), II (pre-fatigued, $F_{100\%}$), III (partially fatigued, $F_{65\%}$), and IV (fatigued muscle, $F_{30\%}$). +

outliers, _ median values, □ mean values. * $p \le 0.0125$ (lines marked with * are connecting data with significant statistical difference). Nonnormalized data

decrease occurred during the protocol, confirming the efficiency of the protocol to generate neuromuscular fatigue in SCI through FES.

Between PoT I and PoT II, muscle fatigue begins to install, although its effects are weakly marked, as both PoTs are statistically equal. While the force intensity persisted at the same level between PoTs I and II, a magnitude reduction (Figs. 6 and 7) occurred in the following frequency bands: 13 Hz (I, 0.66 ± 0.24 a.u., and II, 0.47 ± 0.20 a.u.), 20 Hz (I, 0.66 ± 0.22 a.u., and II, 0.45 ± 0.20 a.u.), and 25 Hz (I, 0.54 ± 0.25 a.u., and II, 0.35 ± 0.21 a.u.). When comparing the reduction in different windows, it became evident that the greatest statistical changes (among protocol instants) occurred at 20 Hz and 25 Hz.

The present study focused on muscle fatigue. FES frequency was set at 70 Hz to quickly induce neuromuscular fatigue, avoiding long FES applications in non-trained muscles and, consequently, in the latter case, possible thermal or chemical burns (Gregory and Bickel 2005; Popovic et al. 2001). However, in future implementation of FES-MMG closed-loop systems aiming to keep the users in the standing position for long periods, different protocols will be necessary to minimize fatigue. When PoT IV is achieved, almost no exercise can be performed, making it very important to detect fatigue prior to this point, e.g., at PoT III.

In healthy subjects, the most relevant MMG frequency bands during submaximal contraction levels are between 12 and 38 Hz (Esposito et al. 1996). Our results show that this

spectral range may also be appropriate for people with SCI. In Figs. 6 and 7, statistical differences between PoTs I and IV were found in all bands between 13 and 35 Hz, except for the 30 Hz frequency band, possibly because of data variability.

The varied responses between different bands in the range of 10–35 Hz for PoTs I and III are noticeable. The magnitude increased at 10 Hz, whereas it decreased at 35 Hz. This is in agreement with results from other studies, which showed that the frequency content shifts towards lower values in the spectrum during the fatigue process (Gorgey et al. 2015). Considering the range between 5 and 13 Hz, we can observe an increase in PoT III, justified by the fact that higher frequencies lead to faster fatigue levels (Binder-macleod et al. 1993).

Comparing non-fatigued muscle (PoT I) with fatigued muscle (PoT IV), decreases with relevant magnitudes were observed in the frequency bands 13, 16, 20, 25, and 35 Hz, as illustrated in Figs. 6 and 7. However, there were statistically significant differences between PoTs I and III only in frequency bands of 20 and 25 Hz. Moreover, the 25-Hz frequency band indicated the greatest significant reduction throughout the neuromuscular fatigue protocol (I, 0.54 ± 0.25 a.u.; II, 0.35 ± 0.21 a.u.; III, 0.33 ± 0.18 a.u.; and IV, 0.31 ± 0.21 a.u.). Regarding the range defined by Esposito et al. (1996), 25 Hz is the central frequency of the range. According to Schillings et al. (2003), neuromuscular fatigue leads to a decrease in nerve speed conduction in SCI individuals, which also occurs in healthy subjects. This event is represented by the vibration attenuation of myofibrils registered



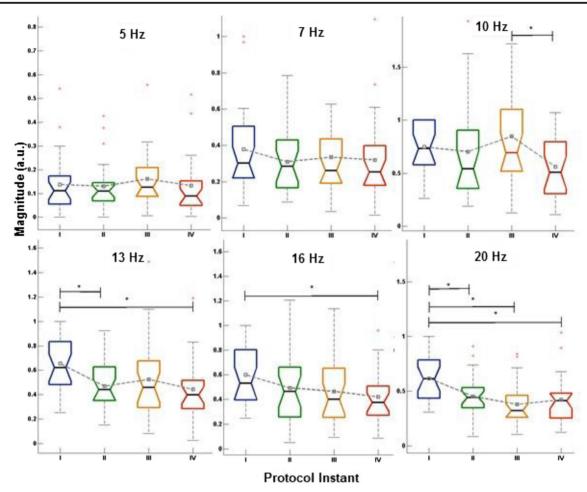


Fig. 6 Boxplot of band frequencies between 5 Hz and 20 Hz during fatigue protocol. Instant: I (non-fatigued muscle, $F_{100\%}$), II (prefatigued, $F_{100\%}$), III (partially fatigued, $F_{65\%}$), and IV (fatigued muscle,

 $F_{30\%}$). + *outliers*, _ median values, \Box mean values. * $p \le 0.0125$ (lines marked with * are connecting data with significant statistical difference). Normalized data

as magnitude reduction, mainly at 25 Hz. However, muscle specificity should be considered, since Beck et al. (2009) found greater intensity at high frequencies in the *rectus femoris* when compared to the *vastus lateralis* and *vastus medialis* muscles.

Researchers have published studies on muscle response during neuromuscular fatigue protocols with other volunteer populations (Kim2015).

Differently, the signals of subjects with SCI decreased in magnitude with neuromuscular fatigue within the 13–35-Hz frequency range, prominently at 25 Hz. After SCI, impaired muscles tend to increase the proportion of fast fibers, which are attributed to causing greater vibration (Gerrits et al. 1999). Thus, the 35-Hz band, which is more associated with fast fibers, should be more susceptible to fatigue and present a marked reduction before the lower band frequencies. Our study did not reveal this event. No pattern was observed of magnitude reduction at high frequencies and magnitude increase at low frequencies, as in neuromuscular fatigue evoked voluntarily (Kim, Corcos, and Hornby. 2015; Kim, Thompson, and Hornby. 2015).

Therefore, one can also hypothesize that the electrically evoked contraction in people with SCI does not follow the same pattern during early FES application as occurs physiologically (Henneman et al. 2017; Jonakait and Street 1994; Gregory and Bickel 2005).

This was an isometric fatigue study, and further investigation is needed to check if the results also apply to dynamic conditions. In that case, it is necessary to use a specific transducer, such as an MIC transducer, which can reduce motion artifacts and ensure proper signal acquisition during movements (Krueger 2014). Zainah et al. (2017) used the MMG sensor to measure torque and correlate it with dynamometer isometric knee torque in SCI subjects, seeking to detect FES-evoked muscle fatigue. The authors found a strong linear correlation, suggesting that the MMG sensor can identify different levels of muscle contraction, which could be extrapolated to muscle fatigue. Focusing on intrinsic muscle changes, our study showed that 20-25-Hz muscle frequencies are more sensitive to the fatigue process, which somewhat complements the results of Zainah et al. (2017).



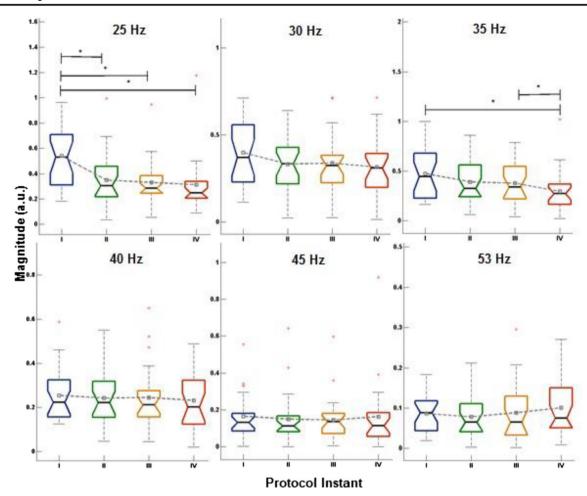


Fig. 7 Boxplot of band frequencies between 25 and 53 Hz during fatigue protocol. Instant: I (non-fatigued muscle, $F_{100\%}$), II (pre-fatigued, $F_{100\%}$), III (partially fatigued, $F_{65\%}$), and IV (fatigued muscle, $F_{30\%}$). + *outliers*, _

median values, \Box mean values. * $p \le 0.0125$ (lines marked with * are connecting data with significant statistical difference). Normalized data

This study could help to detect fatigue more effectively and create new personalized fatigue reduction protocols (Bindermacleod et al. 1993).

Conclusion

The results of this study show that the MMG sensor and wavelet transformation provide direct information about the onset of muscle fatigue. We found the greatest statistical reduction in the transform in the protocol used, at 20–25 Hz. This finding allows the integration of the MMG sensor using a 20–25-Hz bandpass filter as the feedback in future closed-loop FES system processes in order to check the fatigue response.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This research was approved by the Health Ethical Research Committee of the Parana State Institute, protocol number 189/2010, in conformity with the Helsinki Declaration of 1975, as revised in 1983. On behalf of all authors, the corresponding author states that there is no conflict of interest.

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