Optimal force evaluation for isotonic fatigue characterization in mouse Tibialis Anterior muscle

Flavia Forconi
DAHFMO-Unit of Histology and Medical Embryology,
University of Rome La Sapienza, Italy
flavia.forconi@uniroma1.it

Francesca Martelli
Department of Mechanical and Aerospace Engineering,
University of Rome La Sapienza, Italy
francesca.martelli@uniroma1.it

Simona Pisu
Department of Mechanical and Aerospace Engineering,
University of Rome La Sapienza, Italy
simona.pisu@uniroma1.it

Antonio Musarò
DAHFMO-Unit of Histology and Medical Embryology,
University of Rome La Sapienza, Italy
antonio.musaro@uniroma1.it

Emanuele Rizzuto
Department of Mechanical and Aerospace Engineering,
University of Rome La Sapienza, Italy
emanuele.rizzuto@uniroma1.it

Zaccaria Del Prete
Department of Mechanical and Aerospace Engineering,
University of Rome La Sapienza, Italy
zaccaria.delprete@uniroma1.it

Abstract - **Skeletal muscle fatigue is most often studied as a response to repeated stimulations in isometric conditions and it is usually quantified as the progressive loss of force generating capability over time. However, physical dynamic activity is based on the shortening of skeletal muscles. Therefore, the condition that best mimics body movements is the isotonic one, in which muscle is allowed to shorten against a constant load. In the literature, the isotonic fatigue test is performed allowing the muscle to lift a load corresponding to one-third of the maximal isometric force (reference optimal force), as best representative of the force at which the tissue develops its maximum power. The goal of this study was to devise a new testing protocol in which each muscle was tested for isotonic fatigue by shortening against its own optimal force, i.e. the force at which it really developed the maximum power. Our hypothesis was that testing all the muscle at a standard reference value would introduce significant errors in the parameters associated to muscle fatigue and in their variance. The proposed protocol was based on the real-time measurement of the maximum power a muscle was able to generate through the application of the after-load technique and a mathematical interpolation to the Hill's equation, that therefore allowed to determine the experimental optimal force to be applied during the fatigue test. Experimental results showed that the muscles tested with the experimental optimal force had a fatigue time significantly lower than the control muscles tested with the reference optimal force. A decrease, even if not statistically significant, was also measured for the power and work generated during the fatigue test. Of note, for all these parameters a huge decrease in the measurement variance was reported, confirming that a precise assessment of the muscle experimental optimal force was needed to increase the accuracy of the measurements. On the other hand, the application of the protocol proposed in this work required an increase in the test duration, due to the application of the after-load technique, and a real time measurement of the power generated by the tissue.**

Keywords— functional measurements, skeletal muscle, isotonic fatigue, optimal force, maximum power

I. INTRODUCTION

Muscle tissue is characterized by the ability to generate force, to shorten, to produce power and to perform work. However, when muscle is repeatedly stimulated to contract, it develops fatigue. Skeletal muscle fatigue has been widely investigated for several rat, mouse and human muscle types [1,2,3,4,5,6] in isometric condition, which corresponds to a situation where muscle is continuously stimulated and develops force maintaining its length constant. Therefore, muscle force development is the parameter associated with isometric fatigue, which has been defined as the progressive decline in maximum isometric force [7,8]. This isometric behaviour is due to the muscle inability of being able to maintain the expected force over time. However, the condition that best reflects body movements is the isotonic one, in which the muscle shortens against an external load. During such dynamic activity, muscle shortening must be considered together with force, when analysing the development of muscle isotonic fatigue. Therefore, the use of isometric contractions as an experimental model of fatigue is limiting and it should be extended with isotonic ones, which better mimic the fatigue conditions occurring in vivo in muscle activity. Moreover, when a muscle is stimulated repeatedly to shorten against a constant load, it is possible to measure different isotonic fatigue features, such as the work and the power generated by the tissue. To date, in all the studies conducted on mouse [9,10,11] or rat [12,13,14] animal models, the tested muscle was allowed to fatigue by shortening against a load equal to one-third of its maximal isometric force (reference optimal force). This reference optimal force value was chosen as the best representative of the force at which the muscle was capable of generating the maximum power [10,13,15], estimated by the use of the Hill's curve (F-v) [15]. However, even if this value might be a good approximation of muscle optimal reference force, on average, when the difference from the experimental and the reference optimal forces is too high, significant errors might be introduced in all the parameters measured during the fatigue test.

Within this context, the goal of this work was to propose a new experimental protocol for muscle isotonic fatigue, in which each muscle is tested to repeatedly shorten against a load equal to its own optimal force, previously computed through the application of the after-load technique and an interpolation to the Hill's model. To this aim, we conducted, *in situ*, fifteen experiments on Tibialis Anterior (TA) muscles from wild-type mice. During the isotonic fatigue protocol, a group of muscle was stimulated to shorten against the experimental optimal force, and a second group lifted the reference optimal force (one-third of maximal force). Fatigue time, power and work developed by muscles during isotonic fatigue, and the variance of these parameters have been evaluated to assess the accuracy of the two protocols.

II. MATHERIALS AND METHODS

A. Experimental Procedure and testing system

All the experiments were conducted within the animal welfare regulations and guidelines of the Italian national law D.L. 04/03/2014, n.26, about the use of animals for research. Fifteen male and female wild-type mice of 2 to 3 months of age were employed in this study and one Tibialis Anterior (TA) muscle was tested for each animal. In this work, muscle contractile capability has been evaluated through the *in situ* methodology to test the specimens in an experimental condition as close as possible to in vivo one.

At the beginning, the mouse to be tested was anesthetized with an intraperitoneal injection of Ketamina Cloridrato (Ketalar) and during the experiment an extra dose was given if necessary. After removing the skin to expose the hind limb muscles, the tibialis was identified and its tendon was cut a few millimetres far from the end of the muscle, taking care not to include the tendon of the extensor digitorum longus (EDL) muscle in the surgical isolation. Once placed the mouse on a temperature controlled plate (37 ± 1 °C), the hind limb was inserted in a clamp to immobilize it as much as possible and the foot was scotch-taped to the platform. During the experiment, the exposed muscles were kept moist by periodic applications of mineral oil [16]. The TA tendon was tied with a 0.16 mm diameter nylon wire slip knot as close as possible to the muscle attachment and connected to the level-arm of the dual mode Aurora Scientific Instruments 305C-LR actuator/transducer system, as shown in Fig. 1. In particular, the level-arm could be controlled either in force or in position mode, allowing to continuously switch between isometric and isotonic stimulation without interruptions in the experimental protocol.

The actuator/transducer motor was controlled by a custom-made software developed in LabVIEW 2012 through the use of a National Instruments DAQ (PCIe-6363X). The software allowed to set all the experimental parameters necessary to perform the desired protocol, and muscle shortening, force, time derivative of force, shortening velocity and pulse intensity were continuously acquired and stored in text files for post processing.

Muscle contractility was evoked by membrane electrically stimulation by using two wire electrodes (AS632 Cooner Wire), inserted just under muscle surface. Electrical pulses of about 7 mA and a width of 1 ms were generated by a pulse stimulator (701C Aurora Scientific), synchronized by the control software developed in LabView. A digital oscilloscope (Tektronix DPO2014B) was included in the experimental set-up for a real-time visualization of the measured force and length.

For each experiment, the initial muscle length was adjusted to the optimal length (L_0), which produced the highest twitch force [11]. At the end of the test, the mouse was sacrificed by cervical dislocation to minimize suffering. Length and weight of the tested muscle were measured for data normalization through an analog calliper, with an accuracy of 0.05 mm, and a Pioneer precision scale (Ohaus, Parsippany, NJ), with an accuracy of 0.1 mg, respectively. Then, the muscle cross sectional area (CSA) was estimated as follows:

$$CSA\ (mm^2) = m(mg)\ /\ (L_f(mm) * d(mg\ /\ mm^3)) \quad (1)$$

where m is the muscle mass, L_f is the optimal fiber length and d is the density of mammalian skeletal muscle, which is $1.06\ \frac{mg}{mm^3}$ [17]. In particular, L_f is obtained as the product between L_0 and the fiber length to the Tibialis length ratio, which is equal to 0.6, as reported in literature [17,18].

B. Experimental Protocol

To evaluate the error introduced by testing each muscle at the reference optimal force (F_{ref}) rather than at the experimental optimal force (F_{exp}), we subjected two groups of mice to the same experimental protocol, except for the isotonic fatigue phase. In particular, group 1 was constituted by 8 muscles stimulated to shorten against the experimental optimal force, while group 2, made up of 7 muscles, lifted the reference optimal force.

Initially, single pulses were delivered to compute muscle kinetic parameters. After a resting time of 25 s, the muscle was maintained isometric and stimulated with two 0.3 s pulse trains: the first train was delivered at a frequency of 60 Hz, to allow for muscle settlement, while the second one at a frequency of 150 Hz evoked the muscle maximum force (tetanic force). A resting time of 180 s was imposed to allow the muscle to completely recover its functionality. The third phase of the experimental protocol consisted in the application of the after-load technique for the measurement of the maximum power. In particular, five 0.3 s pulse trains at 120 Hz were applied to the muscle, controlling the load that the tibialis had to lift through the actuator/transducer. The resistive load values were set at 30%, 10%, 20%, 80% and 60% of the tetanic force measured during the previous part of the protocol, in such a random order to avoid muscle adaptation to increasing or decreasing loads. Maximum shortening velocity was measured during each isotonic stimulation in order to determine the Hill's curve (F-v) [15], the power curve and, therefore, the experimental optimal force. The protocol concluded with isotonic fatigue measurement: after a recovery of 600 s, the muscle was repeatedly stimulated in isotonic conditions with a series of 0.3 s pulse trains s at a frequency of 120 Hz with a rest time of 1 s before each train. As stated above, the first group of muscles was allowed to shorten against the experimental optimal force (F_{exp}), and the group 2 against the one-third reference optimal force (F_{ref}). The fatigue test ended when the muscle shortening reached the 10% of the maximum value, as shown in Fig. 2. The fatigue time was measured as the time necessary to reach this shortening value. We decided to choose a well determined condition to end the experiments to increase repeatability of the measurement. Immediately after each experiment, tibialis muscle length and weight were measured for the evaluation of its cross sectional area (CSA), as previously described.

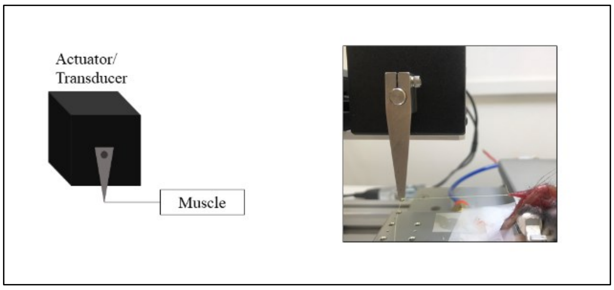

Figure 1: Part of the experimental set-up.

C. Measurement of isometric and isotonic parameters

The data acquisition frequency was set at 1 kHz for pulse train stimulation and at 20 kHz for single pulse stimulation (twitch test). Measurement of maximum force generated by the muscle (F_{max}) was determined immediately after the isometric contraction at the tetanic frequency (150 Hz) and was employed to obtain the values of force to be used during the after-load technique phase. Hill's relationship and power curve were computed once the after-load phase was concluded, through a home-made software developed in LabVIEW 2012. The relationship between muscle force (F) and shortening velocity (v), with F ranging from 0 to 80% of tetanic force, is represented by the Hill's curve [15]. This curve was computed by interpolating the five experimental data of force and shortening velocity obtained at the end of after-load test, using the Levenberg-Marquardt algorithm on the following hyperbolic equation:

$$(F + a) * (v + b) = c \tag{2}$$

where a, b, c are constant values. In addition to the interpolation on the five experimental points, the curve was forced to pass through the point $v = 0$ when $F = F_{max}$. Once the Hill's curve was obtained, the power delivered by the muscle (W) was obtained as the product between the resistive load and shortening velocity values over the entire range of forces, and the experimental optimal force (F_{exp}) was computed as the force value corresponding to the maximum power.

Finally, the power and work generated by the muscle during the fatigue test were computed as the sum of the product of the constant load both for the highest shortening velocity and for the displacement during each shortening, respectively.

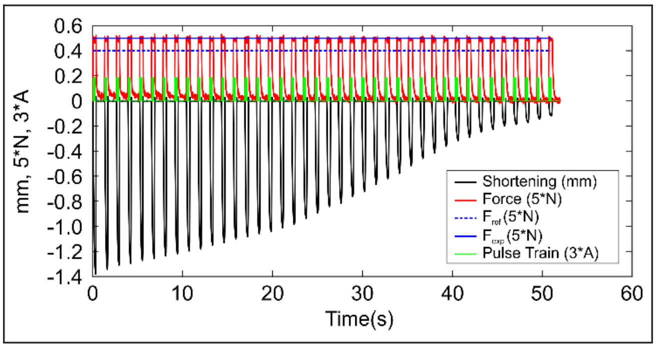

Figure 2: Example of isotonic shortening (black) when muscle shortened against the experimental optimal force (blue line). It is also reported the reference value of the optimal force (dashed blue line).

D. Statystical analysis

Differences in isometric parameters, fatigue time, mechanical work and power of the two tested groups, were evaluated with unpaired t-test. Statistical analyses were performed with GraphPad Prism 6.0 and differences were considered significantly when p-value was lower than 0.05. Values are expressed as mean ± SD.

III. RESULTS AND DISCUSSION

A. Kinetics paramters and specific tetanic force

Tab. I reports mean value ± SD of time derivative during contraction (dF/dt) and relaxation ($-dF/dt$), and absolute (F_{max}) and specific tetanic force (F_{spec}). As expected, isometric parameters of group 1 were not significant different from that of group 2.

B. Power curve

The power curves for TA muscle related to two single experiments are shown in Fig. 3 as an example. For both tests, the maximum power was generated by the muscle at a force level higher than the reference one, namely one-third of the corresponding tetanic force (F_{ref} = 33% of F_{max}) (dashed line in Fig. 3). However, whether in one case the experimental optimal force (37%) was quite close to the reference one, in the other case it was extremely higher (44%) than that. As a result, if both muscles were tested to shorten against the reference optimal force, the second muscle would have been tested in a condition far from that able to allow the generation of maximum power.

Of note, in all of the experiments conducted in this work, the force value corresponding to the maximum power delivered by the muscle was higher than the theoretical one. The experimental optimal force (F_{exp}) was, on average, equal to 39.46 ± 1.4 %.

These preliminary results clearly highlighted the difference between the theoretical and the experimental optimal forces, confirming the need of an optimization of the isotonic fatigue protocols proposed in the previous studies.

C. Isotonic fatigue time

The average values of fatigue time measured for the two muscle groups are shown in Fig. 4. Interestingly, the average fatigue time measured for the muscles tested at the experimental optimal force (group 1) was significantly lower than the value obtained for the group of muscles tested at the reference optimal force (group 2). Reference and experimental optimal force values for the 8 muscles of group 1 are reported in Tab. II.

TABLE I. MEAN ± SD VALUES OF ISOMETRIC PARAMTERS OF GROUP 1 AND GROUP 2.

	Isometric parameters			
	dF/dt (mN/ms)	$-dF/dt$ (mN/ms)	F_{max} (mN)	F_{spec} (mN/mm^{2})
Group 1	5.75±2.08	-3.19±1.13	295.00±115.00	56.81±17.25
Group 2	7.23±5.10	-2.49±0.43	263±44.15	50.55±11.48

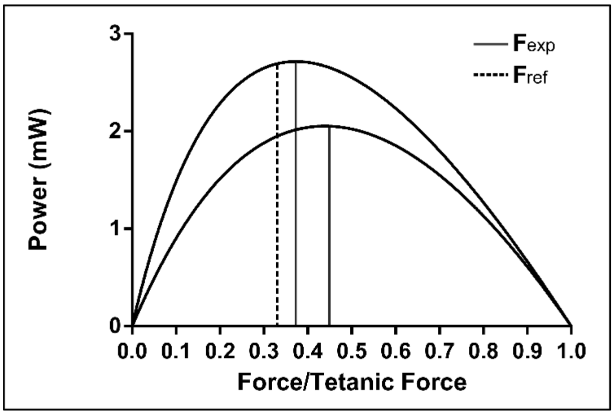

Figure 3: Example of power-force curves for TA muscle related to two experiments.

Since for all the tested muscles the reference force was lower than the experimental one, the specimens tested to shorten against this resistant load seemed more resistant to fatigue. In particular, an increase in the fatigue time of about 95% on average was reported in this case (141.5 ± 86.90 s at the reference force and 72.8 ± 13.4 s at the experimental optimal force). It is worth noting that the fatigue time obtained when testing the muscles to contract against the experimental optimal force showed a high reduction in the variance, in comparison to the group tested at the reference optimal force, and the coefficient of variation (CV) decreased from 61.4% to 18.4%. Indeed, a higher accuracy in the measurement of the fatigue time was obtained when testing each muscle at its optimal force value during the fatigue phase.

D. Isotonic mechanical power and work

The mechanical power and work generated by the muscle during the isotonic fatigue development were computed for each stimulation phase, in which the muscle was able to shorten against the imposed load. In particular, these parameters were obtained by multiplying the load both for the maximum shortening velocity and shortening measured during each contraction.

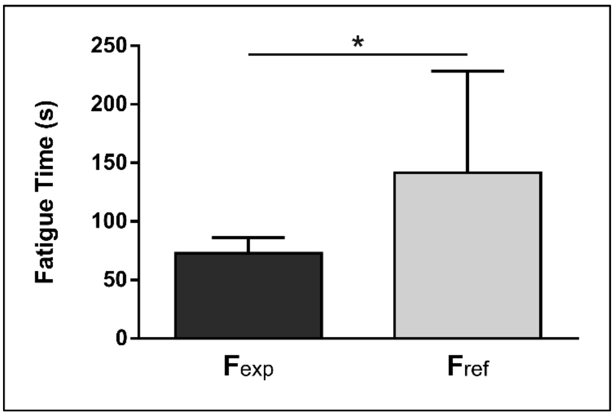

Figure 4: Tibialis Anterior muscle fatigue time measured during isotonic fatigue protocol, when muscles shortened against a load equal to F_{exp} (black) and to F_{ref} (grey). Values are mean ± SD. *: p-value<0.05.

TABLE II. VALUES OF EXPERIMENTAL AND REFERENCE OPTIMAL FORCE FOR THE TIBIALIS ANTERIOR MUSCLE TESTED AT THE EXPERIMENTAL OPTIMAL FORCE (GROUP 1).

	Isotonic fatigue resistive load	
	Experimental optimal force (mN)	***Reference optimal force (mN)***
Test 1	132.15	114.79
Test 2	109.88	92.95
Test 3	127.15	107.21
Test 4	74.68	66.19
Test 5	96.62	77.74
Test 6	77.04	64.73
Test 7	101.14	84.24
Test 8	225.81	182.75

The sum of power and work values computed for each isotonic fatigue trial and normalized to TA muscle weight are shown in Fig. 5 and 6, respectively.

Results related to power and work were in line to what already found for the fatigue time. In fact, a decrease of both power and work was reported when testing muscles from group 1, even if not statistically significant. Once again, a huge reduction of the variance in the measurements was reported, with CV values lowering from 69.24% to 33.10% and from 57.55% to 24.49% for power and work, respectively.

These results confirmed that the use of the experimental optimal force allowed to obtain more accurate measurements in all the parameters of interest during the isotonic fatigue phase.

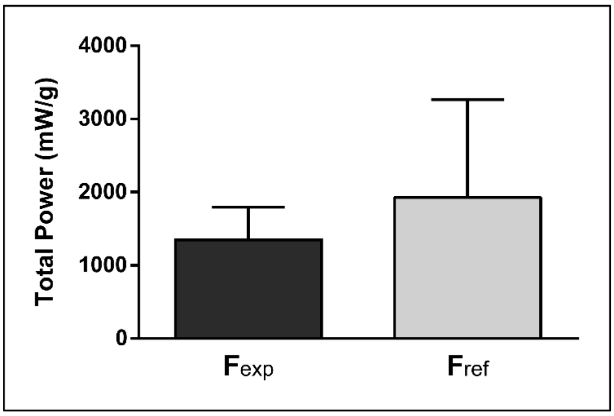

Figure 5: Normalized total mechanical power during the development of isotonic fatigue. Values are mean ± SD.

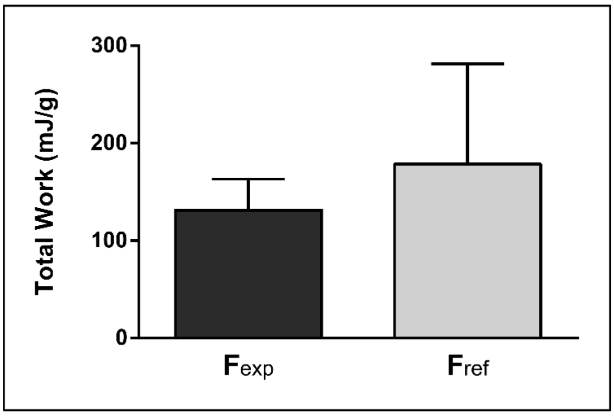

Figure 6: Normalized total mechanical work during the development of isotonic fatigue. Values are mean ± SD.

IV. Conclusions

In this work, we developed and validated an experimental protocol to evaluate *in situ* the isotonic fatigue of wild-type mouse Tibialis Anterior muscles tested at their experimental optimal force. To this, a real-time measurement of the maximum power generated by the specimen was carried out before the beginning of the fatigue test. A comparison with the results obtained with standard isotonic test performed at the reference optimal force (1/3 of maximum force) was performed.

Of note, in all the tests conducted in this work, the experimental optimal force was higher than the reference one. As a consequence, the fatigue time was lower when testing the muscles to load their experimental optimal force; indeed, when stimulating a muscle to continuously lift a lower load, the muscle seemed to be more resistant to fatigue. Interestingly, the fatigue time as well as the power and work generated during the fatigue test showed a lower variance when the muscle were tested at their experimental optimal force. It has to be noted, that the proposed protocol required the measurement of the maximum power generated by the muscle before the beginning of the fatigue test, being this not necessary if testing the muscle to fatigue at the reference optimal force. However, the increase in the experimental duration and in the control software complexity were necessary to highly enhance the accuracy of the measurements during this essential contractile test.

Finally, the experimental protocol here devised might be even more crucial when testing pathologic or transgenic mice, whereas the experimental optimal force might be even more different from the reference one.

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