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HAL Id: hal-03676721

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Passive and active mechanical tests at different scales of the skeletal muscle: a literature review

Revue de la littérature sur les tests mécaniques passifs et actifs aux différentes échelles du muscle squelettique

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ABSTRACT. Human skeletal muscle is a complex tissue with a strict and ordered hierarchy (muscle, fiber, myofibril) similar to rodent animal used to study the mechanical properties of healthy and pathological muscles (e.g. mdx mouse to mimic Duchenne disease). Collagen envelopes, actin and titin are the structures implicated in the passive mechanical properties. The active mechanical properties are related to the formation of actin-myosin cross bridges. This article presents the most commonly used mechanical tests to measure *in vitro*, at different scales, the passive (incremental stepwise extension test, stretch-release test, compressive test, fatigue-recovery test, eccentric contraction test) and active (force-frequency test, tetanus and twitch contraction tests) behaviors of rodent muscles. The next section of this literature review covers the need for *in vivo* protocols to be as close as possible to physiological conditions, allowing to keep the animal alive and to perform longitudinal mechanical studies, with the presentation of imaging methods (MRI and ultrasound-based elastography) in living rodents. Then the main factors (protocol heterogeneity, aging, etc.) influencing the mechanical properties are presented.

RÉSUMÉ. Le muscle squelettique humain est un tissu complexe et hiérarchisé (muscle, fibre, myofibrille) similaire à celui du petit rongeur utilisé pour étudier les propriétés mécaniques des muscles sains et pathologiques (par exemple chez la souris mdx qui modélise la myopathie de Duchenne). Les enveloppes de collagène, les filaments d'actine et de titine sont les structures impliquées dans les propriétés mécaniques passives. Les propriétés mécaniques actives sont reliées à la formation des ponts actine-myosine. Cet article présente les tests mécaniques les plus couramment utilisés pour mesurer *in vitro*, à différentes échelles, les comportements passifs (test d'étirement progressif incrémental, test d'étirement-relâchement, test de compression, test de fatigue-récupération, test de contraction excentrique) et actifs (test en force-fréquence, test avec des contractions courtes et longues) du muscle chez le petit animal. La section suivante de cette revue de la littérature couvre la nécessité de protocoles *in vivo* pour être au plus près des conditions physiologiques, permettant de maintenir l'animal en vie et ainsi de réaliser des études mécaniques longitudinales, avec la présentation de méthodes d'imagerie (élastographie par ultrasons et par IRM) chez des rongeurs vivants. Enfin, les principaux facteurs (hétérogénéité des protocoles, vieillissement, etc.) influençant les propriétés mécaniques sont présentés.

KEYWORDS. skeletal muscle, mouse, passive and active mechanical properties, *in vitro* and *in vivo* mechanical tests, ultrasound and MRI elastography.

MOTS-CLÉS. muscle squelettique, souris, propriétés mécaniques passives et actives, tests mécaniques *in vitro* et *in vivo*, élastographie par ultrason et par IRM.

1. Introduction

1.1. Rodent muscle model

Skeletal muscle is a complex tissue with a strict and ordered hierarchy [BEN 06] so as the tendon [GUM 10] and the bone [BEN 04]. Through its contractile activity, it is the effector of the movement and posture of a subject through its attachment to the bone via the tendons. Like any organ of the body, many factors can influence its general state such as ageing [BUT 06], pathologies [MCD 95], muscle training [THO 94], and other internal and external factors to the subject.

The muscle tissue of many animals can have an organization (actine-myosin cross bridges, fibril, fiber, collagenous membranes, etc.) and a genetic composition (for instance 90 % for rodents) as almost similar to that of human muscles. The rodent model was especially used for a long time to

study the mechanical properties of skeletal muscle, and it was a good solution for the study of neuromuscular disorders, especially Duchenne muscular dystrophy [LAR 14], [PAS 93]. Even today the rodent is widely used in muscle characterization as further presented in this review. The strength of this model is related to its short life span allowing the study of long-term phenomena, such as aging for example. Another advantage is the speed of development of a line thanks to the speed of reproduction of rodent species, as well as the huge panel of genetic lines available for the study of many pathologies.

However, some differences are observable between the skeletal muscles of human and rodent. For instance, the proportions of slow and fast muscle fibers differ between these two species [SPA 03]. According to the same authors, differences in adaptation to physical effort are also observed between the rodent models and human with a difference in expression of the type of fiber according to the physical training followed. Moreover, Burkholder & Lieber [BUR 01] have showed in 2001 changes in the sarcomere length between between the Human (2.64 μm), the rat (2.4 μm) and the rabbit (2.27 μm). However, despite these specificities, several studies showed that the mouse represents a good model for the analysis of human mitochondrial activity [JAC 12], which is important in the process of muscle activation, especially for slow oxidative fibers. Thus, the use of rodents remains one of the most effective means for the further study of muscle biomechanics. The rodent (mice, rat) animal allow: (1) an easier accessibility of muscle samples, to study the mechanical behavior at different levels (myofibril, fiber, muscle), (2) the analysis of genetic muscle pathologies (e.g. mdx (muscular dystrophy X-linked) mice for Duchenne muscular dystrophy [AND 02]), and (3) the analysis of the effect of treatment at different scales of the muscle. Then, the challenge will be to transpose the animal results to the human. In the present article, we will summarize the different mechanical tests performed on rodent muscle at different scales.

1.2. Multi-scale structure

Its hierarchical structure, as shown in Figure 1, implies a compartmentalized organization at several scales. The complete muscle, representing the macroscopic scale, is enveloped in a layer of matrix tissue called epimysium. The microscopic scale corresponds to an isolated muscle fiber surrounded by the endomysium. Between the two is the mesoscopic scale, represented by the muscle fascicle which is delimited by the perimysium. This mesoscopic scale allows for the separate activation of different groups of fibers for better management of the energy required to activate the muscle, depending on the type of movement performed. The different layers separating these three scales represent the extracellular matrix (ECM) [FRO 15].

Each fiber is composed of myofibrils where a large number of longitudinal filaments (titin, actin, myosin) (Figure 1) are either involved in passive and/or in active states [ODD 09]. Transversal filament, such as desmine, is aligned in a transverse manner through the Z-disks of the sarcomere.

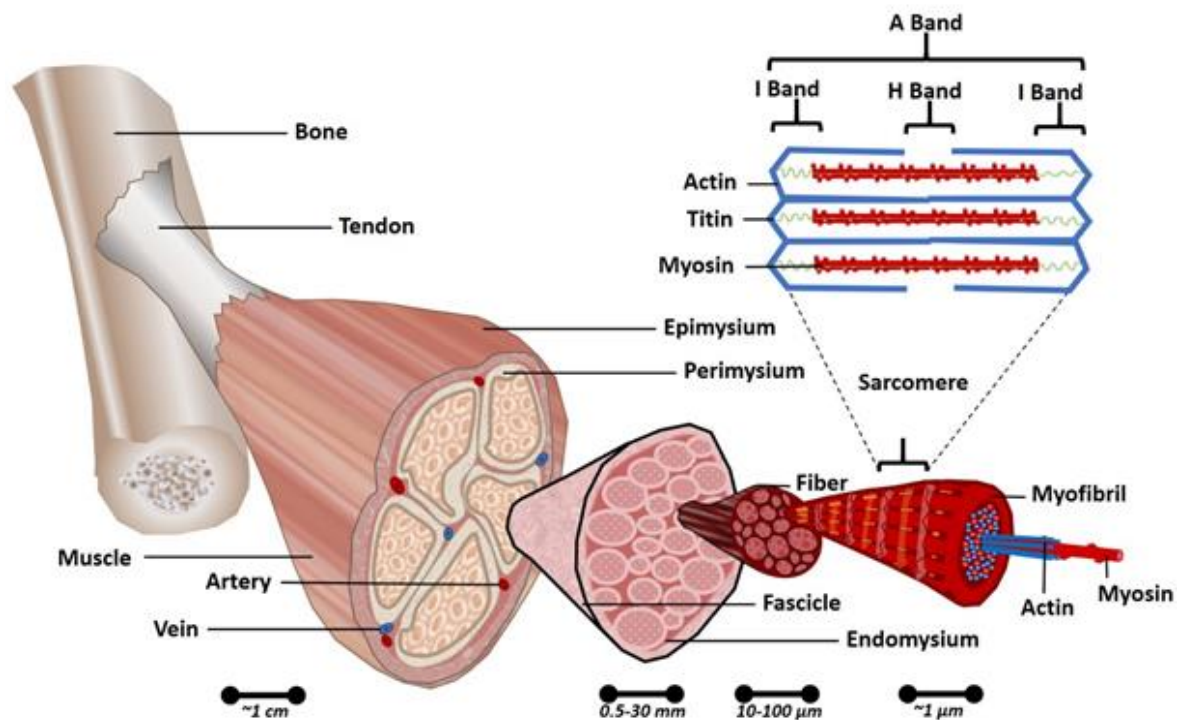


Figure 1. Multi-scale architecture of skeletal muscle.

1.3. Structures related to passive mechanical properties

Skeletal muscle has passive mechanical properties related to the ECM structure presents at different scales [ROW 10]. These collagen envelopes are mainly composed (70 %) of collagen fibers (type I, III) with a surrounded structure made of proteoglycans, glycoproteins and elastin. In addition to allowing the force transmission between the collagen fibers, they also provide elasticity of the entire muscle. Indeed, type I is organized on parallel fibers and provide to the muscle its stiffness while type III is organized on a loose mesh and confers to the tissue its compliance [KOV 02].

In addition to the ECM, the titin filament is a molecular spring inside sarcomeres (Figure 1) that spans each half-sarcomere from the Z-line to the M-line and is known to produce most of the passive force in isolated sarcomeres and myofibrils [GRA 00], [JOU 08], [POU 21]. Titin isoforms vary across muscles and account for the differences in passive force between myofibrils harvested from different muscles [HOR 92], [KON 09], [LIN 00], [LIN 18], [POW 17].

1.4. Structures related to active mechanical properties

The sarcomere, the smallest contractile unit of the muscle (Figure 1), is composed of myosin and actin filaments. The formation of actin-myosin cross bridges, via a cascade of chemical (Adenosine Triphosphate (ATP), Adenosine Diphosphate (ADP), inorganic phosphate, calcium) reactions allow a shortening of the sarcomere, inducing the muscle to contract [BLO 11] and the force development [CAR 17]. Depending on the type of myosin (I or II), the muscle fibers is defined as oxidative (type I inside slow muscle such as soleus), or glycolytic (type II inside fast muscle such as the extensor digitorum longus (EDL)). The oxidative fibers require less activation energy to contract, thus allowing slow and prolonged activity, and using the Krebs cycle to synthesize the ATP molecules. Conversely, the glycolytic fibers, require high activation energy to contract, allowing an explosive and short activity, and will use the cycle of glycolysis to synthesize ATP molecules. Each skeletal muscle has a different proportion of type I and II fibers depending on the activity it is used for (type I are more resistant to the fatigue than type II). In the literature, the soleus and the EDL are often characterized due to their oxidative and glycolytic composition, respectively [HOP 06], [PET 97], [PET 00], [SCH 11], [SCO 01], [STA 90].

1.5. Plan of the article

In this article are presenting the most commonly used mechanical characterization tests present in the literature. We first explain the tests used for the characterization of the passive properties, including the description of the tests themselves, the measured parameters, and the different applications. The second part is focused on the active mechanical characterization, while the third part presents *in vivo* tests in development these last years, mainly based on elastography methods. Then a last part on the parameters influencing the mechanical properties is developed before the conclusion and the perspectives part of this present review.

2. *In vitro* mechanical characterization

2.1. Sample preparation

As a preliminary to any form of testing, the preparation of rodent muscle samples requires special attention and precautions. At the level of the skeletal muscle, *in vitro* characterization consists of extracting the muscle from its physiological environment for study. During the dissection, the muscle is sutured at each tendon and then attached to a mechanical testing device composed of a force transducer that records the force developed passively or actively by the muscle, and a removable platform to modulate the length of the muscle. Throughout the experiment the muscle is immersed in an oxygenated physiological solution (Krebs [HES 19], Ringer [SMI 14], Tyrode [MIT 15]) mimicking the natural environment of the muscle to have the most realistic results possible. The muscle contraction is mainly induced by electrical stimulations using electrodes placed in the bath where the muscle is attached. At the level of the muscle fiber, the samples are prepared from the muscle which is kept for 12 hours at 4°C in a skinning solution [POU 21]. Then, muscles were washed in a series of graded glycerol concentrations (12.5, 25 and 50 %) prior to storage in a 50/50 glycerol/skinning solution at -20°C.

2.2. Passive mechanical characterization

In this paragraph, incremental stepwise extension (ISE) test and stretch-release test will be presented to characterize the passive elements of the skeletal muscle.

2.2.1. Incremental stepwise extension test

The muscle is placed at its slack length (L_s), which corresponds at the length where the muscle starts to develop a measurable resting tension, of around few millinewtons (5 mN: [ROW 10], or 10 mN: [CAN 08], [TOS 10]). Other studies have placed the muscle at its optimal length (L_o), where sarcomeres are at their length where they develop maximum contraction force (around 2.4 μm for rodents) [BUR 01]. The muscle is then dynamically stretched at different percentages of its initial length (L_s or L_o) and hold until a relaxation plateau is reached without any electrical stimulation.

From this test, elasticity and viscosity are determined with the dynamic and static stress parameters recorded and corresponding to the peak stretch and the steady state, respectively. Relaxation times are also studied for the analysis of the viscous behavior [AND 02]. In the same way, ISE test can also be applied at the level of the muscle fiber, where the initial length of the muscle fiber is defined at the sarcomere length (2.4 μm [JOU 08]).

ISE was applied for the analysis of aging to analyze the muscle elasticity in function of age (rat: from 1 to 24 months) [CAN 08]. Studies have demonstrated that aging increases the stiffness of oxidative muscle from less than 0.451 MPa at 1 month old to nearly 0.97 MPa at 24 months old [CAN 08]. The changes in elasticity with age appear to be much more important in young than in old rats, this being correlated with the modification of collagen type and content.

ISE is also applied to analyze specific muscle structure involved in passive behavior such as the desmin filament, located in the transversal direction, and the titin filament, oriented longitudinally. The characterization of these structures is of importance for myopathy (muscle disease) such as desminopathy and titinopathy. In their studies of 2001 and 2002, Anderson et al. [AND 01], [AND 02] have demonstrated that desmin knockout mice showed an increase of the dynamic and passive muscle stiffness. This result revealed the importance of this transversal filament to maintain the passive mechanical behavior of the muscle. Concerning the titin filament, genotypes with shorter size titin filaments leads to more than doubling of the dynamic and passive muscle stiffness, as on desmin knockout (KO) mice [BUC 14], [HA 18]. However, Ha et al. [HA 18] explained that their results diverged from previous studies [GIL 11], [LIE 17], [MEY 11] by showing that titin protein plays a greater role on ECM passive mechanical properties. A better understanding of the mechanical behavior of these passive structures will allow to follow the effect of treatments.

ISE test can be used for other genetic disease such as Duchenne myopathy [HAK 11], [HAK 12], [HAK 13]. Mice with Duchenne myopathy (mdx mice) had higher dynamic stiffness (750 kPa) compared to control (450 kPa), or wild type (WT) mice [HAK 13], and a modification of the rupture point of the musculo-tendinous junction (MTJ). In addition, results showed that the impact of this myopathy on passive mechanical properties are age and sex dependent. The analysis of relaxation times showed that they were higher on mdx mice, demonstrating that viscous properties were also affected. Because of its fibrosis profile, the authors [BRA 21], [SMI 14] used mdx mice model, to study what can impact passive mechanical properties of the ECM. For the first time, they have shown on 2014 that changes are not linked to the quantity of collagen on the ECM. And then, in 2021, they showed that passive properties are linked to the collagen fibers architecture. Their mechanical experiments were accompanied with optical analyses to see collagen packing and hydroxyproline dosage (which is an indicator of collagen presence).

The comparison of the mechanical results between the studies are difficult due to the variability of the experimental parameters (initial length, percentage of stretch, velocity of the stretch, etc.) used by each study. Figure 2 shows an example of the changes of the mechanical result, showing that Buck et al. [BUC 14] have obtained lower stiffness values than Hakim's study [HAK 11].

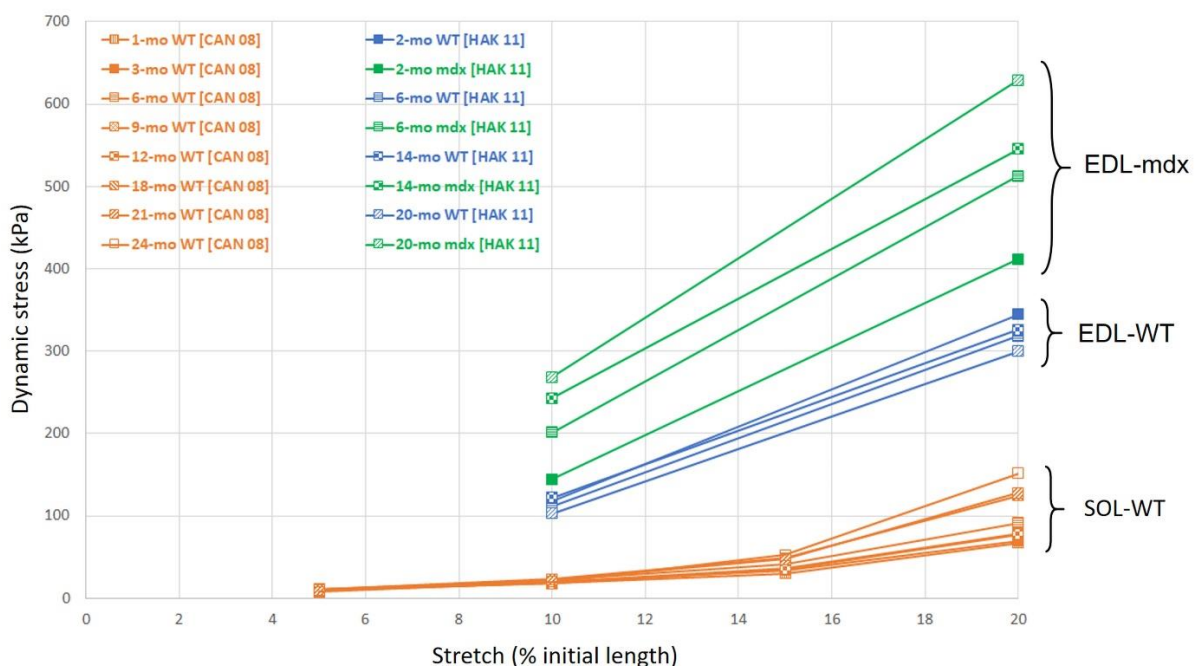


Figure 2. Dynamic stress in soleus (SOL) and extensor digitorum longus (EDL) at different months (mo) of age induced by incremental stepwise extension [CAN 08], [HAK 11]. Orange, blue and green colors are used for wild type (WT) soleus of rat, wild type EDL of mice and mdx (muscular dystrophy X-linked for Duchenne Dystrophy Muscle) EDL of mice, respectively.

We can notice that there are many possible representations for the results of this test. One of these representations is, as shown on Figure 2, standardized by cross-sectional area (CSA, mm²) for whose formula is [DEL 08]:

$$CSA = \frac{m}{L_f \cdot 1.06} \quad [1]$$

where L_f (mm) is the optimal fiber length related to the optimal length (L_o) of the muscle, m (mg) the muscle mass, and 1.06 (mg.mm⁻³) is the mammalian skeletal muscle density.

Another solution is to represent results of absolute force as in Anderson et al. [AND 02].

2.2.2. *Stretch-Release test*

The muscle is stretched to a percentage of its initial length (L_s or L_o) at a constant speed ($0.1 \text{ mm}\cdot\text{s}^{-1}$) and then released at the same speed to its initial length [CAN 08]. From this test is determined the tangent modulus in the linear part of the stress-strain curve, as well as the stiffness at peak stretch.

In this study referred above, it was used to analyze the evolution of the passive mechanical properties of the muscle over ageing of the animal from 0.75 month to 24 months old, showing an increase of the muscle stiffness with age of both the tangent modulus and the passive stiffness at peak stretch. This test was also used to determine the impact of maternal undernourishment, on the passive properties of the muscle of the offspring [TOS 10]. Undernutrition during the fetal period leads to an increase in these parameters revealing an increase in passive muscle stiffness, in nascent rats.

The stretch-release test can also be applied on muscle fiber [BEN 06] to compare the elastic range of value between the macroscopic and microscopic level.

2.2.3. *Compressive test*

Compressive tests are scarcely used in the literature compared to the current traction. However, it is important to characterize the transversal direction to have a better understanding of the role of the transversal structure such as the transversal desmine filament or the collagen cross link behavior. The passive characterization tests presented in the literature are mainly in simple traction. However, it is possible to find mechanical compression tests, as in the study of Leichsenring et al. [LEI 21]. This study applied compressive test on soleus muscle from rabbits and they have demonstrated age-dependent variations in mechanical properties. At the level of the muscle fiber, atomic force microscopy was performed and the transversal cartographies of elasticity have revealed defect in the mechanical properties [KAM 19].

Similarly, we also find some studies where dynamic stretching tests are used via sinusoidal mechanical stimulation in the studies of Moran et al. [MOR 05] whose protocols were taken from Gordon et al. [GOR 88] and Stein et al. [STE 86] on the study of the evolution of the passive properties of the muscle, in a mouse model, over the course of life.

2.3. *Active mechanical characterization with electrical stimulation*

The active characterization protocols are performed by inducing electrical stimulation of the muscle via electrodes on each side of the muscle. They allow the study of microstructures and biological processes involved in the process of sarcomere shortening. The isolated muscle will use the micro-components present in the physiological solution in which it is immersed to contract. There are four main tests used in the literature: the force-frequency relationship test, the twitch and tetanic supramaximal contraction test, the fatigue resistance and recovery test, and the eccentric contraction test.

2.3.1. Force-Frequency test

The force-frequency relationship test is often used in the literature to characterize the active behavior of the muscle, i.e., during a contraction induced by the shortening of the sarcomeres. The muscle is placed at its optimal length L_0 , in order to obtain the maximum force developed during a long tetanic contraction. The aim is to induce long tetanic contractions at different frequencies. The recording of the force developed by the muscle at each stimulation allows the force-frequency relationship curve to be plotted. This test can be represented in two ways, providing different information. Firstly, it allows the contractile activity of the muscle to be characterized by quantifying the behavior of specific type of fiber present in the muscle. At the lower frequency, the slow fibers are more recruited than the fast fibers. As in figure 3, the curve will be represented by the percentage of the maximum developed force, depending on the frequency of activation. It can be noted that the study of Moran et al. [MOR 05] allows us to differentiate between a slow muscle (soleus) and a fast muscle (EDL). The developed force, whatever the age of the mouse, reaches its plateau at around 120 Hz of stimulation frequency for the fast muscle (EDL), whereas the slow muscle (soleus) reaches it at lower frequency around 60-80 Hz, for healthy mice. The frequency information is particularly useful for other active tests as the fatigue / recovery and eccentric contraction test. Secondly, if we plot the graph as a function of absolute force or specific force (with respect to the CSA), we notice differences in developed force. Thus, there is a degradation of the contractile components in the soleus of aged mice, with a maximal tetanic force going from 160 mN in 4-month-old mice to less than 130 mN in 28-month-old mice. Similarly, Flexor Digitorum Brevis (FDB) muscle were used to characterize the contractile properties of skeletal muscle in comparison with soleus and EDL muscle [TAR 18]. In a more pathological setting, studies have used mdx mice to characterize the contractile properties of muscle which degrade with age [BAR 05], [HAK 13]. They have demonstrated that the study of contractile properties is a good indicator of disease progression (from a specific EDL tetanic force greater than 200 kPa for healthy mice, and less than 150 kPa for mdx mice). From these results, Barton's study [BAR 05] shows the beneficial muscle effect of several weeks of arginine treatment for mdx mouse while another study [LIU 05] demonstrated the effects of injection of a virus mediating micro-dystrophin expression in young mdx mice. Force-frequency test is currently used by many studies to analyze the impact of inhibition of (1) gene on muscle development [SIT 14], (2) apoptotic activity [MIT 15], (3) a muscle mass regulator [DHU 20], and (4) titin protein [BUC 14] on the contractile properties of muscle. Each study showed a decrease in maximal tetanic force developed by pathological muscles compared to healthy muscles which can be accompanied by a fast to slow fiber switch for soleus muscle [SIT 14].

In addition, other studies use this force-frequency test to observe the recovery of the contractile system by short- or long-term treatment. For instance, the effects of creatine intake on muscle fatigue [HEA 11] and the recovery of the activity of the SERCA (sarco/endoplasmic reticulum Ca-ATPase: transport of Ca^{2+} to sarcoplasmic reticulum from the cytosol) [QAI 19] showed an increase in the maximum tetanic force developed by the muscle.

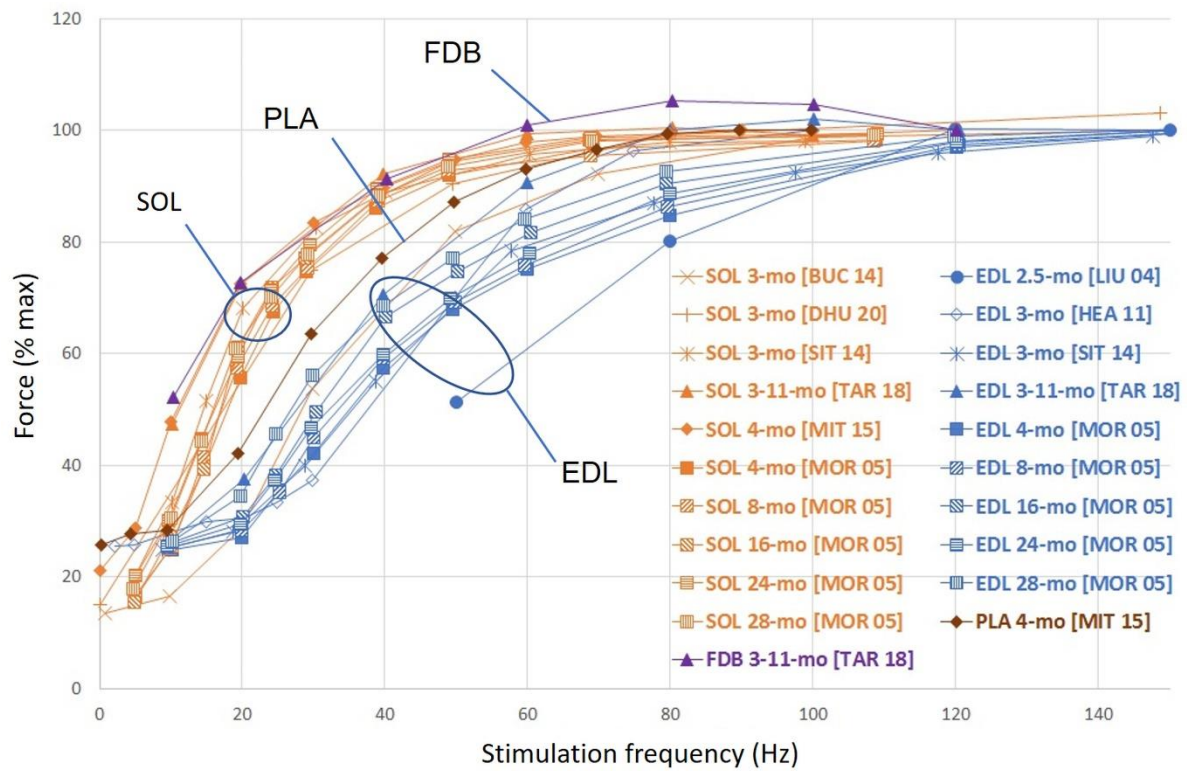


Figure 3. Force-frequency results for four wild type (WT) muscles from female mice of different months (mo) of age from literature [DHU 20], [HEA 11], [LIU 04], [MIT 15], [MOR 05], [SIT 14], [TAR 18]. Max tetanic force (%) is plotted along stimulation frequency (Hz). Orange, blue, brown and purple colors represent the behaviors of the soleus (SOL), the extensor digitorum longus (EDL), the plantaris (PLA) and the flexor digitorum brevis (FDB) muscles, respectively.

2.3.2. Supramaximal (tetanus) and instantaneous (twitch) contraction tests

Instantaneous twitch contractions and long tetanic contractions provide complementary information of the muscle mechanical properties. Indeed, Buck et al. [BUC 14] showed significant differences for a long contraction and not for a short one, and vice versa. It may therefore be interesting to observe the tetanus and the twitch responses for the characterization of the same muscle.

The supramaximal contraction test consists of electrical stimulation of the muscle at a high enough frequency to activate all the fibers in the muscle. From its optimal length, the muscle is subjected to an instantaneous contraction (named twitch) of a few milliseconds. This test allows the measurement of different parameters such as the time to peak force (TPF) or time to peak tension (TPT), the half relaxation time (HRT) and the maximum stress (MS) or developed tension. These parameters are known to characterize the calcium cycle during a contraction, and are respectively related to the sequestration of calcium in the sarcoplasmic reticulum for the TPF, and to its reabsorption for the HRT [KHO 15], [ROB 10]. MS is related to the excitation-contraction relationship [ROB 10]. Therefore, a change in calcium release activity leads to an increase in the rate of force development and relaxation of skeletal muscle [ØRT 00]. The activity of Adenosine Triphosphate of the SERCA pump does not appear to be related to contractile activity since its restoration does not result in changes in HRT and TPT for the soleus muscle [QAI 19].

As we can see on Figure 4, slow and fast muscles have different characteristics mainly due to their different contractile proteins (related to the different types of myosins). Indeed, Barton's study [BAR 05] shows that a healthy soleus has a HRT and a TPF more than twice higher than an EDL for the same age (3 months old mice). Moreover, the maximum force is about 2.5 times higher for an EDL muscle than for the soleus, while if we look at the maximum stress with the force normalized

with the CSA, there is only 24 kPa more for the EDL compared to the soleus. These results are of the same order of magnitude as those found by Moran et al. [MOR 05] on the impact of aging on the contractile properties of mouse muscle on 4-month-old mice, showing also that EDL muscle tends to become « slower » with an increase in HRT (about 8 ms) between 4 and 28 months. However, the MS of the soleus decreased by about 70 kPa between 8 and 28 months.

Tetanus test, corresponding to a long muscle contraction, was performed on mdx soleus and EDL [SMI 14], [BRA 21]. The results show an increase for the HRT, TPF, and the tetanic specific tension. These changes are related to an increase in muscle mass (and therefore CSA) despite a constant absolute force. In addition, Brashear's study [BRA 21] demonstrated that the HRT value increases for the soleus of mdx mice compared to controls. The same trend of « slowing down » for the EDL is found in the studies by Hakim et al. [HAK 12], [HAK 13].

The involvement of the titin filament in the muscle contractile system, playing a role in active force development, was demonstrated [HER 08], [HER 14], [LIN 18]. Buck's study [BUC 14] used a mice model IG (immunoglobulin) with titin mutation to show a small decrease in the tetanic TPF and a slight increase in the twitch HRT for the soleus. In case of mdm (muscular dystrophy myositis) mice model [HES 19], different results were found on soleus with a shorter time of contraction, a HRT more than 3-fold higher than normal mice, and a 2-fold decrease in force development rate.

This test enables also to characterize fasting on the muscle contractile properties. Kvedaras's study [KVE 20] shows a non-significant tendency to increase HRT, but significantly decrease the specific twitch force developed by aged mice subjected to fasting.

Changes in fiber type within a muscle can occur in case of weightlessness, training, rodent model (MGs mice: Gs α -deficient inducing fast-to-slow-fiber-type switch) etc. inducing changes in contraction kinetics. Feng et al. [FEN 11] show that the MGs mice model, revealing an increase in TPF and HRT parameters, and a decrease in twitch and tetanic force.

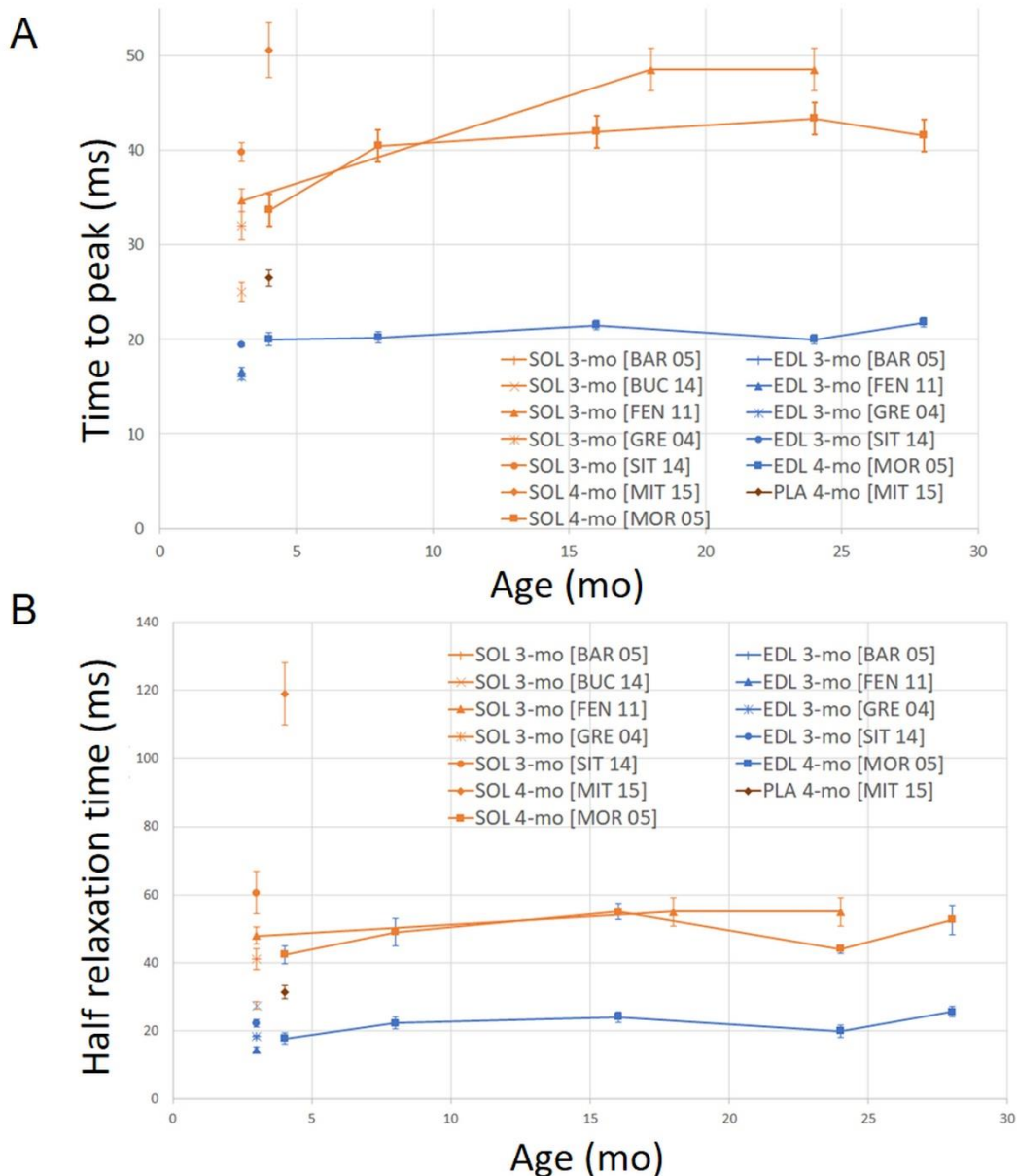


Figure 4. (A) Time to peak tension and (B) half relaxation time results for three wild type (WT) muscles from female mice of different months (mo) of age from literature [BAR 05], [BUC 14], [GRE 04], [MIT 15], [MOR 05], [SIT 14]. Orange, blue and brown colors represent the soleus (SOL), the extensor digitorum longus (EDL) and the plantaris (PLA) muscles, respectively.

2.3.3. Fatigue and Recovery tests

The test is mainly used to study phenomena related to muscle fatigue and is also called Work Loop Cycles (WLC) [JAM 04]. The muscle is placed at its optimal length (L_0) and then electrically stimulated (from 50 Hz to 80 Hz) to induce long and repeated contractions over time. The increase in muscle fatigue is characterized by a drop in the tetanic force developed. Many assumptions were studied to physiologically explain the decrease of the force. Non-exhaustively, we can find the concentration of lactic acid, potassium and hydrogen ion accumulation, or the accumulation of inorganic phosphate in the sarcoplasm [WAN 17]. The physiological mechanisms studied by Ørtenblad et al. [ØRT 00] put forward that the release of calcium from the SR is impacted by exposure to repeated contractions, which may explain the decrease in this contraction force. Zhang et al. [ZHA 06] have tested the changes in fatigue resistance in fast muscle (EDL) and slow muscle (soleus) in the presence of K^+ ion, lactic acid and cyanide. Despite the fact that these three compounds are known to induce an increase in fatigue, only the presence of cyanide (inhibitor of mitochondrial electron transport) had an effect on both muscles by decreasing the strength. In

addition, a dependence on temperature in the fatigue process was demonstrated. Moreover, Head et al. [HEA 11] observed an increase in strength during a fatigue test related to the presence of creatine, reducing ionic forces.

The WLC test was applied to healthy and transgenic mice to study the fatigue resistance in different muscle diseases. The fatigue protocol used in the studies of Mitchell et al. [MIT 15] and Sitparan et al. [SIT 14], described in part 2.3.1 on the force-stimulation frequency test, show respectively no significant difference in force remaining after 5 min between pathological and healthy animals, and a decrease in fatiguability in EDL and soleus for the pathological mice.

Feng et al. [FEN 11] also showed an increase in fatigue resistance in MGs (G protein α -subunit ($G_s\alpha$)) mice which have a glucose intolerance with a switch from fast to slow fibers that can be observed during the process of fatigue. However, despite an increase in fatigue resistance in EDL muscle, this regulator does not appear to play an important role in the contractile properties or fatigue resistance.

Despite being a commonly used test, comparing results between different studies remain difficult as many experimental parameters of the fatigue protocol can vary the rate at which muscle strength drops. For instance in case of healthy EDL (mice, 4 month), Sitparan et al. [SIT 14] found a 70 % loss of strength in 4 min compared to 30 s obtained by Head et al. [HEA 11]. This may be explained by the fact that the protocol of Head et al. [HEA 11] applied a tetanic contraction during 1 s every second compared to a stimulation of 0.7 s every 5 s for Sitparan et al. [SIT 14].

2.3.4. *Eccentric contraction-injury (ECI) test*

From its optimal length L_0 , the muscle is subjected to tetanic stimulation, and followed by passive stretching. The contractions are repeated over time as in a fatigue test, and result in a decrease in the tetanic force developed at each contraction. Eccentric contractions are known to result in muscle micro damage [HOD 19]. Analysis of muscle regeneration mechanisms following damage was investigated by Call and Lowe [CAL 16]. In addition, ECI is used to investigate the impact of training on the adaptation of the muscle to damage. Thus, Capogrosso et al. [CAP 17] have showed that unlike healthy mice, the mdx mice did not show adaptation to training. To compensate for this loss of strength in mdx profiles, several authors have tested numerous treatments. For instance, Liu's study [LIU 05] tested the injection of a virus mediating the expression of a microdystrophin, leading to an increase in resistance to damage.

ECI was extensively performed on Duchenne myopathy (mdx mice) in multiple studies [BAL 11], [CAL 16], [CAP 17], [LIU 05], [MAR 09], [OLT 18], [SON 09], and it was demonstrated that the mdx tissue is more sensitive to damage from eccentric contractions with an average force loss of 80 % compared to over 10 % for a profile of healthy muscle (Figure 5). This result is explained by the deficiency of the dystrophin protein which helps protect the muscle [BAL 11] not present in mdx mice muscle. In the same way, overexpression of the γ -cytoplasmic actin in mdx mice showed an increase in protection to force drop in the study of Baltgalvis et al. [BAL 11] and Olthoff et al. [OLT 18] by leading to an inhibition of peroxiredoxin-2 which is an oxidative enzyme. This benefit is also found in Galgt2 overexpressed mdx mice where an average recovery of 80 % of the loss of strength regardless of age was observed [MAR 09]. Moreover, a difference of almost 100 % of the loss of strength was shown for healthy mice, regardless of age. The same type of result on mice overexpressing utrophin, a dystrophin homolog, were observed [SON 09].

However, the comparison of ECI results between the studies follow the same tendency. As can be seen in Figure 5, different behaviors for healthy muscles, submitted to the same conditions (training, sedentary), were published [MAR 09], [CAP 17]. The force differences can be explained by the method of attaching the muscle to the device [MAR 09], the speed of stretching or even the rest time between each contraction [OLT 18].

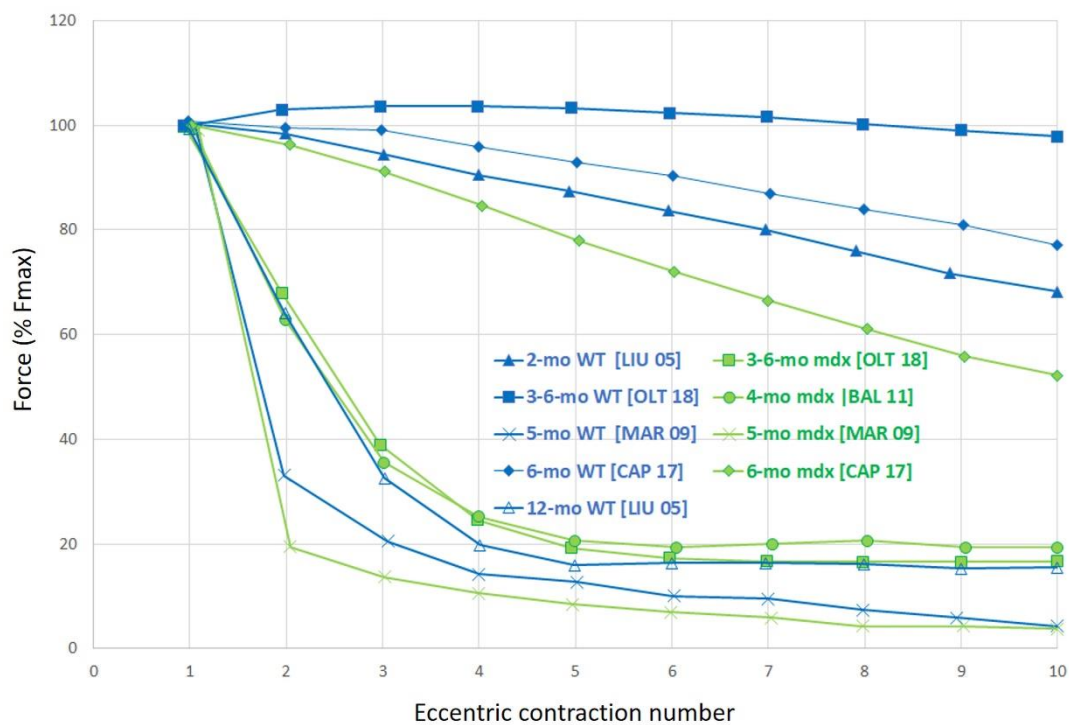


Figure 5. Force loss results during eccentric contractions in percentage of maximal tetanus force for extensor digitorum longus (EDL), from female mice of similar months (mo) of age, in function of genotype (WT: wild type, mdx: muscular dystrophy X-linked for Duchenne Dystrophy Muscle), from literature [BAL 11], [CAP 17], [LIU 05], [MAR 09], [OLT 18]. Blue and green colors are used for WT and mdx genotypes, respectively.

One of the main advantages of *in vitro* experimentation is the ability to control the metabolites and their concentrations present in the test environment. In addition to the metabolites used by the muscle, other components can be introduced to disrupt muscle metabolism and cause muscle dysfunction. This makes it possible to easily test several experimental conditions that would be difficult to test *in vivo*.

3. *In vivo* mechanical characterization

In addition to *in vitro* characterization, some studies have analyzed the muscle with *in vivo* method. The set up and protocols are essentially the same as *in vitro*, with the difference that the muscle remains in its physiological environment (the animal's paw) with the supply of micronutrients and oxygen via its own bloodstream. The muscle contraction is operated by electrical stimulation directly on the nerve of the target muscle. These differences in experimental conditions (*in vitro* vs *in situ*) can lead to changes in the mechanical behavior of the skeletal muscle. Another point that can be really useful on the use of *in vivo* experiments is the accessibility of longitudinal experiment, by keeping animals alive and makes experiment at different ages on the same animals.

Actually, there is a strong need for non-invasive, *in vivo*, imaging techniques which enable longitudinal follow-up in live animals with muscle diseases and in the understanding of fundamental biological processes. Magnetic Resonance Elastography (MRE) and Shear Wave Elasticity Imaging (SWEI) are methods that can meet future needs.

3.1. Magnetic Resonance Elastography (MRE)

MR-elastography method aims at measuring the viscoelastic properties of tissues *in vivo* in a non-invasive way. MRE is based on the generation of stationary harmonic shear waves within the organ, their encoding by MRI imaging and the reconstruction of the viscoelastic mechanical properties of

the soft tissue [MUT 95]. This method allows the assessment of the mechanical properties of biological soft tissues in a non-invasive way, as close as possible to their physiological, healthy or pathological conditions. This imaging modality is increasingly used in clinical research as well as in clinical routine for medical diagnosis, including in muscle, in patients with hyperthyroidism Duchenne muscular dystrophy [BEN 07], [BEN 15], or simply in the characterization of muscle from childhood to adulthood [DEB 11].

MRE was used occasionally in preclinical studies on different organs of rodent as varied as the liver [EVE 20], [YIN 07], [ZHU 17] or brain [ATA 08], [BIG 18]. Concerning the muscle, the application of MRE was first proposed on rodent skeletal to investigate the relevance of muscle mechanical anisotropy as a biomarker of tissue necrosis [QIN 14], showing that, when the rate of necrotic tissue increases, the mechanical anisotropy ratio decreases. More recent studies have investigated the differences between healthy and pressure ulcerated muscle in rats, showing an increase in shear modulus of the tibialis anterior muscle from 4.2 kPa to 5.1 kPa [NEL 17], [NEL 19]. However, the use of MRE technique in the pre-clinical field in rodent model remains scarcely studied.

3.2. Ultrasound Shear Wave Elasticity Imaging (SWEI)

Similarly, SWEI allows an analysis of skeletal muscle stiffness by assessing shear modulus with the propagation of shear waves through the muscle tissue [GEN 13], [COS 13]. Few authors propose to apply SWEI to characterize the elastic properties of skeletal muscle in rodents. For instance, Kammoun et al. [KAM 19] and Ternifi et al. [TER 20] have developed specific hindlimb experimental protocols using SWEI to characterize the passive stiffness of muscle transgenic mouse model to better understand the impact of a gene (KLF10 or TIEG1) on the muscle elasticity (Figure 6). In addition to the study of Kammoun et al. [KAM 16], the link between muscle mechanical properties and pathological microstructure was proposed by Martins-Bach et al. [MAR 21] by studying the differences in stiffness in mice with different levels of collagen in matrix tissues, allowing to analyze the increase in collagen level during muscular fibrosis.

Changes in muscle stiffness linked to the ECM were recently studied [MAR 21] by SWEI method on fibrotic mice genotypes, and showed an increase of average stiffness (S) when the paw of mice is placed at 40° ($S_{\text{injured}} = 34.8$ kPa vs $S_{\text{control}} = 27.8$ kPa) and 60° ($S_{\text{injured}} = 44.0$ kPa vs $S_{\text{control}} = 34.0$ kPa) of ankle plantar flexion.

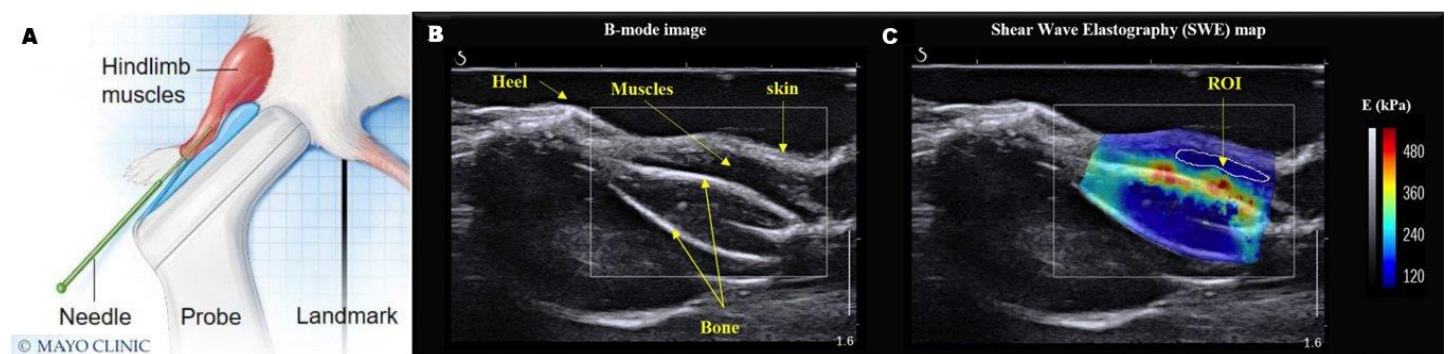


Figure 6. (A) Ultrasound set up, (B) B-mode image of mice hindlimb, (C) cartography of elasticity (E : Young modulus) with a region of interest (ROI) composed of 3 muscles (soleus, gastrocnemius, peroneus brevis) (reprinted from [KAM 19] and [TER 20], with permissions from Elsevier and Springer Nature).

4. Influencing parameters on mechanical properties

The passive and active mechanical behaviors of muscle are dependent on many parameters. Inter-individual variations, set up of the protocols, experimental parameters, or muscle metabolisms are all conditions that can lead to mechanical differences between the studies.

4.1. Protocol heterogeneity

In the literature, there is no two identical mechanical protocols, leading to different results. Thus, the comparison of data between studies raised numerous questions about the experimental conditions, such as in case of ECI test: how long the muscle is stimulated? At which frequency? To which stretch is it subjected? How long is the rest time between each stimulation? Olthoff et al. [OLT 18] have tested different rest time (3 and 30 min) between 2 contractions and they have showed an influence of the rest time on the decrease of the muscle force.

In case of the fatigue protocols, Sitparan et al. [SIT 14] and Feng et al. [FEN 11] have published different fatigue behaviors for healthy oxydative (soleus) and for glycolytic (EDL) muscles. It is assumed that these variations may be related to the conditions of the electrical excitations, i.e. the duration of the contraction, the frequency, the rest time between contractions, and the number of contractions.

4.2. The aging

Aging is characterized by morphological and functional changes on the skeletal muscle. These changes are involved in the modification of passive (increase of stiffness) and active (decrease of contractility) mechanical properties, a switch of muscle fiber, an increase of collagen tissue, etc. [CAN 08], [ROS 07]. Thus, it is important to compare muscle data from mice with the same age. For instance, Moran et al. [MOR 05] have demonstrated a decrease of the maximal tetanic force developed by the muscle on aged mice for the soleus and an increase of the half relaxation-time for the EDL. In the same way, Feng et al. [FEN 11] have showed that the fatigue resistance and recovery increase as a function of age.

4.3. Other impacting factors

Skeletal muscle has a large number of genes involved in its development and maintenance of its integrity throughout life. In case of genetic disease, it was demonstrated that the muscle mechanical properties were affected. For instance, Hakim et al. [HAK 11] showed the changes of the passive mechanical properties for EDL in Duchenne myopathy (mdx mice). Other factors such as the nutrition [KVE 20] and the physical activity [CAP 17] are also parameters which can also impact the mechanical behavior of the muscle.

5. Conclusions and perspectives

The mechanical characterization of skeletal muscle was largely developed in the last decades. In a pre-clinical context, the rodent model became essential in the study of neurodegenerative pathologies such as Duchenne muscular dystrophy, old age, physical training, or in the development of medical treatments. Many *in vitro* tests were developed, and used in the literature, to characterize the passive behavior (such as viscous, elastic or hyperplastic) and the contractile behavior (tetanic force, time to peak force, relaxation time). However, the *in vitro* test conditions partially mimic the physiological environment of the muscle, which can create biases in the recorded properties. This is why new non-invasive imaging methods of *in vivo* characterization were developed in recent years. MR and ultrasound elastography methods, commonly used in human clinical studies (liver, ...), are promising techniques and will be further developed for the *in vivo* and non-invasive analysis of mechanical properties of the skeletal muscles in rodent models.

ACKNOWLEDGEMENTS

This study was funded by the Research Department of UTC within the framework of AMI International. This work was also supported by French state funds managed within the “Plan Investissements d’Avenir” and by the ANR (reference ANR-10-IAHU-02), Labex CAMI ANR-11-LABX-0004 and the ITI HealthTech.

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