

# MUSCLE MECHANICAL PROPERTIES OF ADULT AND OLDER RATS SUBMITTED TO EXERCISE AFTER IMMOBILIZATION

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## ABSTRACT

**Objectives:** To describe the effects of immobilization, free remobilization and remobilization by physical exercise about mechanical properties of skeletal muscle of rats of two age groups. **Methods:** 56 Wistar rats divided into two groups according to age, an adult group (five months) and an older group (15 months). These groups were subdivided in: control, immobilized, free remobilized and remobilized by physical exercise. The pelvic limb of rats was immobilized for seven days. The exercise protocol consisted of five swimming sessions, once per day and 25 minutes per session. The gastrocnemius muscle was subjected to tensile tests, and evaluated the properties: load at the maximum limit, stretching at the maximum limit and

stiffness. **Results:** The immobilization reduced the values of load at the maximum limit and the remobilization protocols were not sufficient to restore control levels in adult group and older rats. The stretching at the maximum limit differs only in the older group. **Conclusions:** The immobilization reduces the muscle's ability to bear loads and exercise protocol tends to restore the default at control values in adult and older rats. The age factor only interfered in the stretching at the maximum limit, inducing a reduction of this property in the post-immobilization. **Level of Evidence II, Investigating the Results of Treatment.**

**Keywords:** Muscle, skeletal. Immobilization. Biomechanics. Age factors.

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## INTRODUCTION

The immobilization of a body segment is a procedure generally used for the treatment of musculoskeletal injuries, although it can result in undesirable structural alterations<sup>1</sup> such as muscle atrophy,<sup>2</sup> change in the number of sarcomeres in series,<sup>2</sup> reduction in the glycogen reserve,<sup>3</sup> increase of connective tissue, diminished strength<sup>2,3</sup> and muscle weight.<sup>4</sup>

Previous studies showed alterations after two or three weeks of immobilization,<sup>3,4</sup> yet there are studies that show that some of these adaptations can already be observed in less than seven days.<sup>2,5</sup>

Williams et al.<sup>6</sup> declare that starting from six hours of immobilization, it is possible to observe loss of proteins. There are reports that the muscle develops atrophy after 48 hours of immobilization and that 37% of its weight is reduced after seven days. The intensity with which muscle adaptations occur is directly influenced by the position in which the limb is immobilized.<sup>7</sup> Herbert and Balnave<sup>8</sup> studied the effects of different immobili-

zation positions from plantarflexion through to complete dorsiflexion and concluded that the immobilization of a muscle in the stretched position delays the disuse atrophy process; however, the muscle immobilized in the shortened position, induces more intense alterations.

As regards aging, it is known that this process is characterized by diminished function in the tissues, organs and systems of the body, with reduction in the ability to adapt to internal and external stimuli.<sup>9</sup>

In the skeletal muscle, aging provokes progressive decrease in weight, force generation and regeneration capacity, together with an increase in susceptibility to injuries, oxidative stress and inflammatory process.<sup>10,11</sup>

Aging is also associated with the progressive reduction of motor neurons, generating deficient innervation in the musculature. Although many fibers are reinnervated by other motor neurons, this process is insufficient to totally compensate for alterations caused by the denervation, resulting in the atrophy of muscle fibers.<sup>12</sup>

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The deleterious effects of immobilization associated with the alterations provoked by the senescence process on the skeletal muscles can result in greater susceptibility to injuries, which makes the study of its responses to external stimuli an interesting topic.

Few studies evaluate the method used to process the recovery of muscle resistance after immobilization, and particularly, immobilization followed by remobilization.<sup>13-17</sup> Thus, the evaluation of the mechanical properties of the muscle tissue is an important procedure, consisting of a very useful tool for the establishment of clinical/surgical protocols and rehabilitation programs, as it provides relevant knowledge about the possible muscle adaptations and alterations in the presence of external stimuli.<sup>17</sup> It is also difficult to find studies that address these processes in the aged skeletal muscle.

In light of the aforesaid considerations, this study aims to describe the effects of immobilization, free remobilization and remobilization by physical exercise on the mechanical properties of the muscle tissue of rats from two age brackets.

## MATERIAL AND METHOD

### Animal experimentation subjects

The study subjects were 56 male Wistar rats (*Rattus norvegicus*) supplied by the Central Vivarium of Universidade Estadual Paulista (UNESP) – Botucatu Campus, SP, and kept in the vivarium of the Histology and Histochemistry Laboratory of Presidente Prudente (FCT/UNESP). The animals were housed in collective polypropylene cages with four rats in each one, under controlled conditions of temperature ( $22 \pm 2^\circ\text{C}$ ) and humidity ( $50 \pm 10\%$ ) and light/dark cycle of 12 hours (7-19h), with water and feed *ad libitum*.

All the adopted procedures were approved by the Committee of Ethics in the Use of Animals (CEUA) of the Faculdade de Ciências e Tecnologia de Presidente Prudente (FCT/UNESP) under protocol no. 05/2010.

### Experimental groups

The animals were divided into two groups according to age bracket, with one adult group aged five months (*Group A*) and one older group aged 15 months (*Group B*). These were randomly subdivided into four experimental groups:

- Adult control (A1,  $n = 7$ ) and Older control (B1,  $n = 7$ ).

Animals that remained in the vivarium during the experimental period and were euthanized in a paired manner with the remobilized groups.

- Adult immobilized (A2,  $n = 7$ ) and Older immobilized (B2,  $n = 7$ )  
Animals that underwent plaster cast immobilization and were euthanized soon after its removal.

- Adult free remobilized (A3,  $n = 7$ ) and Older free remobilized (B3,  $n = 7$ ).

Animals that underwent plaster cast immobilization, followed by free remobilization, after which they were euthanized.

- Adult remobilized by physical exercise (A4,  $n = 7$ ) and Older remobilized by physical exercise (B4,  $n = 7$ ).

Animals that underwent plaster cast immobilization followed by two days of free remobilization and physical exercise in the form of swimming over a period of five days, and were subsequently euthanized.

### Immobilization technique

The animals were anesthetized with ketamine hydrochloride (80mg/kg) and xylazine hydrochloride (15mg/kg) applied intraperitoneally<sup>5</sup> and upon conclusion of the anesthesia plan, had their ankles wrapped in tubular mesh, followed by the application of quick drying plaster bandages with a width of approximately three centimeters, for bilateral immobilization of the hind legs, from the pelvis to the ankle. It was decided to position the hind legs in extension (pelvis, hip and knee) and the ankle in maximum plantarflexion, which kept the gastrocnemius in a shortening position.

The animals remained immobilized for seven consecutive days<sup>2,5,16,18</sup> and were kept in individual cages with unrestricted access to water and feed.

The plaster was changed when necessary, respecting the same procedure.

### Free remobilization

Upon removal of the cast, the animals from groups A3 and B3 were placed in collective cages for free remobilization, remaining in the vivarium for a seven-day period, after which time they were euthanized.

The animals from groups A4 and B4 also underwent the same procedure, but for a period of two days, before the application of the physical exercise protocol.

### Adaptation to the fluid medium

Prior to the application of the plaster cast immobilization technique in the animals from groups A4 and B4, these were submitted to a fluid medium adaptation process.

The adaptation occurred in a cylindrical tank with a smooth surface, measuring 120cm in diameter by 75cm in height, with a water level of 10cm and water temperature kept at  $31 \pm 1^\circ\text{C}$ . The animals were placed in this tank, staying there for 15 minutes/day over 10 consecutive days.

The purpose of the adaptation was to reduce the animal's stress without, however, promoting physiological adaptations resulting from the physical exercise.

### Physical exercise protocol

After two days of free remobilization, the animals from subgroups A4 and B4 underwent five individual (daily) swimming sessions in a tank measuring 120cm in diameter x 75cm in height, divided into eight cylindrical compartments of PVC (30cm in diameter x 120cm in height) containing water at a depth of 70cm and temperature of  $31 \pm 1^\circ\text{C}$ . There was no addition of load in the animals and each session lasted 25 minutes.

### Collection and preparation of the material

The animals were euthanized using an overdose of ketamine hydrochloride and xylazine hydrochloride applied intraperitoneally,<sup>17,19</sup> following the principles of ethics in animal research. The right gastrocnemius of each animal was excised by means of the removal of the skin and of some soft parts, taking the precaution to maintain its integrity, preserving the origin in the distal third of the femur and the calcaneal insertion to facilitate the fixation of the piece to the testing machine.

After the dissection, the muscle was placed in a Ringer's lactate solution, at room temperature, until the performance of the tensile tests, a period of less than one hour.<sup>5</sup>

## Mechanical tensile testing on muscles

For the tensile test the examiners used a universal testing machine (EMIC®, model DL2000) from the Physics, Chemistry and Biology Department of Faculdade de Ciências e Tecnologia de Presidente Prudente - FCT/UNESP, equipped with a load cell with a capacity of 50kgf.

The machine used has a direct interface with a microcomputer, with the Tesc® program, able to generate a graph, of load versus stretching, for each trial.

Two sets of accessories were fashioned for the fixation of the piece to be tested, with one for fixation of the femur and the other for fixation of the calcaneum, keeping the knee and ankle at an angle of 90°.

After the fixation of the muscle to the testing machine, the muscle was submitted to axial traction with a preload of 300g<sup>17</sup> for 30 seconds, with the intention of promoting the system's accommodation.

After preload, the trial proceeded at a pre-established speed of 10 mm/minute. The applied load was recorded by the software at regular stretching intervals until the time of muscle rupture.

The load versus stretching graphs of each trial were used to obtain and analyze the following mechanical properties:

- load at the maximum limit (LML): highest load value recorded before muscle rupture. It is represented in Newtons (N).

- stretching at the maximum limit (SML): maximum stretching reached before muscle rupture. Represented in meters ( $\times 10^{-3}\text{m}$ ).

- relative stiffness: represents the passive resistance of the muscle, being determined by the tangent of the angle ( $\theta$ ) formed by the straight line drawn in the elastic phase, expressed in Newtons/meter (N/m).

## STATISTICAL ANALYSES

The data obtained were submitted to the Shapiro-Wilk test for normality applied using the SPSS 17.0 for Windows statistical program.

The load at the maximum limit (LML) and stiffness data appeared normal; accordingly, the examiners used the analysis of variance (ANOVA-One-Way) followed by Tukey's post-test performed by means of the SPSS statistical program for comparison between the subgroups in each group (A and B).

The stretching at the maximum limit (SML) data appeared non-normal both in group A and in group B. Accordingly, to compare the subgroups, the examiners used the Kruskal-Wallis test followed by Dunn's post-test by means of the InStat program. A significance level of 5% was used for all the analyses.

## RESULTS

Immobilization reduced the load at the maximum limit (LML) values both in the adult and in the older animals (Tables 1 and 2). It can be noted that although the protocol of remobilization by physical exercise shows a tendency to increase the value of this property, this was not sufficient to restore LML at control levels. Free remobilization generated results close to those of the immobilized subgroups, not showing positive effects in the short post-immobilization period.

In relation to stretching at the maximum limit (SML), no significant differences were found between the experimental adult groups.

In the results obtained in the older animals there was a decrease in SML in subgroups B3 and B4.

The studied protocols did not generate significant changes with regards to the property of stiffness.

The mean values and standard deviations of the mechanical properties obtained from the different experimental groups studied are observed in Table 1 (adult group) and Table 2 (older group).

**Table 1.** Values of means and respective standard deviations of the mechanical properties of stiffness, load and stretching at maximum limit of the adult subgroups.

Groups	Stretching at the Maximum Limit ( $\times 10^{-3}\text{m}$ )	Load at the Maximum Limit (N)	Stiffness ( $\times 10^3\text{N/m}$ )
A1	16.67 $\pm$ 4.05	53.49 $\pm$ 2.40 <sup>b,c,d</sup>	4.88 $\pm$ 0.84
A2	12.22 $\pm$ 2.40	41.99 $\pm$ 3.19 <sup>a</sup>	5.21 $\pm$ 1.42
A3	12.46 $\pm$ 1.43	39.59 $\pm$ 4.25 <sup>a</sup>	4.31 $\pm$ 0.78
A4	13.28 $\pm$ 3.02	45.38 $\pm$ 6.21 <sup>a</sup>	4.64 $\pm$ 1.88

<sup>a</sup>p<0.05 (compared with A1); <sup>b</sup>p<0.05 (compared with A2); <sup>c</sup>p<0.05 (compared with A3); <sup>d</sup>p<0.05 (compared with A4). A1 = Adult control; A2 = Adult immobilized; A3 = Adult free remobilized and A4 = Adult remobilized by physical exercise.

**Table 2.** Values of means and respective standard deviations of the mechanical properties of stiffness, load and stretching at maximum limit of the older subgroups.

Groups	Stretching at the Maximum Limit ( $\times 10^{-3}\text{m}$ )	Load at the Maximum Limit (N)	Stiffness ( $\times 10^3\text{N/m}$ )
B1	15.65 $\pm$ 3.14 <sup>c,d</sup>	60.33 $\pm$ 3.96 <sup>b,c,d</sup>	5.03 $\pm$ 1.39
B2	13.07 $\pm$ 2.18	43.86 $\pm$ 2.81 <sup>a</sup>	4.43 $\pm$ 1.22
B3	10.92 $\pm$ 2.05 <sup>a</sup>	42.73 $\pm$ 5.78 <sup>a</sup>	5.43 $\pm$ 1.20
B4	10.94 $\pm$ 2.23 <sup>a</sup>	46.60 $\pm$ 4.81 <sup>a</sup>	5.99 $\pm$ 1.02

<sup>a</sup>p<0.05 (compared with B1); <sup>b</sup>p<0.05 (compared with B2); <sup>c</sup>p<0.05 (compared with B3); <sup>d</sup>p<0.05 (compared with B4). B1 = Older control; B2 = Older immobilized; B3 = Older free remobilized and B4 = Older remobilized by physical exercise.

## DISCUSSION

The present study sought to describe the effects of immobilization on the mechanical properties of the gastrocnemius of rats from two age brackets (five months and 15 months), as well as the responses to the protocols of free remobilization and remobilization by physical exercise.

Several mathematical models were developed to understand the muscle biomechanics, yet their use does not allow an understanding of the biomechanical behavior of the muscles in the presence of intervening variables.

Therefore, the analysis by means of the mechanical tensile test is an interesting option, as it allows an evaluation of the muscle's adaptive response to different stimuli, besides simulating loads imposed on the musculoskeletal structure, which is constantly affected by the action of external forces, especially in the form of tension.

The gastrocnemius was used on account of its anatomical location, which makes it possible to preserve the physiological characteristics concerning muscle origin and insertion after dissection, enabling its fixation to the tensile machine for performance of the tests. This is important, as the analysis of the biological tissue as a whole structure reflects the physiological mechanical solicitation in a more trustworthy manner,<sup>20</sup> besides which the gastrocnemius works under conditions of extreme physical activity and runs an increased risk of injuries and ruptures.<sup>1,21,22</sup>

As immobilization is a precursor of significant muscle alterations, the interest of studying protocols that can minimize or reverse such alterations becomes important. Another point that deserves analysis is the choice of animals from different age brackets (adults and older animals). This option was based on the fact that age, both in humans, and in animals, influences the strength and the change in the measurements of the cross section of a muscle.

Although the physiological process of the muscles is relatively well known, the total days of immobilization, the difficulty encountered by the animals in moving around inside the containment boxes and the remobilization time were weighed in the choice of the model. In view of the above, the definition of the protocol of immobilization and of remobilization of the pelvic limb appears justified.

As the mechanical properties of the muscle are obtained without the action of neural activation, they depend only on their structural composition, since there is no variation in the number of actin and myosin cross-bridges that would interfere in the values of these properties.<sup>23</sup>

In the present study, the rupture site of the muscles submitted to the mechanical tensile test was in the muscular venter. According to Järvinen,<sup>19</sup> when the gastrocnemius is submitted to tensile tests, muscle rupture almost always occurs at the same site, whereas in his study rupture occurred in the muscular venter in 94% of the test subjects. The results found by Lima et al.<sup>2</sup> and Sene et al.<sup>1</sup> corroborate these findings, since they presented the same characteristic with regards to the muscle rupture site.

Lima et al.<sup>2</sup> suggest that the fact that the ventral region of the muscle presents a greater concentration of muscle tissue than of connective tissue can make it more susceptible to rupture. The fact that it is impossible to adequately measure the cross section area during the performance of the tests prompted us to opt to evaluate the data using load instead of *tension*.

The evaluation of stretching at the maximum limit (SML) demonstrates that the immobilization protocol did not induce statistically significant alterations in the adult group. We can only observe a tendency for reduction verified by means of the lower mean values of the subgroups that underwent this process when compared with the control group.

Carvalho et al.<sup>20</sup> observed significant reduction of the values of stretching at the maximum limit in the gastrocnemius, after 14 days of immobilization.

In the older group, significant differences were observed between the control group (B1) and the subgroups free remobilized (B3) and remobilized by physical exercise (B4), presenting a reduction of the SML. There is a visible, yet not significant, tendency for a decrease in SML in the immobilized group (B2). Thus, it can be suggested that the deleterious effects caused by this procedure would have persisted in the post-immobilization period, resulting in lower values of this property. Although it has not been possible to determine which factors could directly influence such a finding, we can suggest that the age factor might interfere in a negative manner in the body's recovery response, since the aging process is related to diminishing function of the biological tissues, specifically in relation to progressive reduction of motor neurons.

The results of load at the maximum limit (LML) demonstrated a

significant decrease in the muscles submitted to immobilization, both in the adult and in the older group. The reduction of the values of this property indicates that the immobilized muscle bears less weight, and therefore becomes more susceptible to injuries.

Järvinen et al.<sup>21</sup> demonstrated alterations in the organization and in the structural characteristics of the collagen fibers, such as increase in the number of perpendicularly oriented fibers, narrower, more numerous and less tension-resistant fibers. Generally speaking, the quantitative and qualitative alterations in the intramuscular connective tissue, resulting from the immobilization, can contribute towards the reduction of the biomechanical properties of the immobilized skeletal muscle.

According to Abdalla et al.<sup>22</sup>, exercise induces the functional alignment of the collagen fibers and Stone<sup>23</sup> asserts that physical exercise can increase connective tissue resistance and muscle mass, making the muscle more resistant.

In the present study, the results showed that the protocol of remobilization by physical exercise was not sufficient to determine the reestablishment of load at the maximum limit at the control level, yet we can observe a tendency to increase, both in the adult and in the older group.

In relation to the subgroups submitted to free remobilization, these presented values that were very close to those of the immobilized subgroups; therefore, this protocol did not present positive responses in the short post-immobilization experimental period.

Carvalho et al.<sup>13</sup> performed plaster cast immobilization of the hind leg of rats for a period of three weeks, either followed or not followed by free remobilization and remobilization by physical exercise for four weeks. The authors noted that both remobilization protocols obtained efficient responses in terms of restoring LML at control levels. These findings could possibly differ from the present study due to the remobilization process exposure times that surpassed that of this study and of its immobilization period.

Muscle stiffness represents an important property to be studied, since the reduction of its values indicates that the muscle is stretching more in the presence of a smaller load, which also renders it more susceptible to injuries.<sup>5,7</sup>

Considering the deformation of structural proteins of the muscle fiber during the mechanical trial, among the structures that are accountable for this tensile resistance behavior, special emphasis should be placed on the extracellular matrix and titin<sup>24</sup>, a structural protein of sarcomere that assists in the natural passive resistance of the muscle. These two structures are considered responsible for the viscoelastic resistance of the musculotendinous complex.<sup>17</sup>

Immobilization reduces the extensibility of sarcomeric proteins (titin) and their isoforms ( $\alpha$  and  $\beta$ )<sup>2</sup>, besides promoting modifications in the extracellular matrix.<sup>17</sup>

However, in this study, the immobilization protocol was probably not sufficient to cause changes in this property both in the adult group and in the older group.

Carvalho et al.<sup>15</sup> found reduction of stiffness, load and stretching at the maximum limit resulting from immobilization for 14 days. The free remobilization process over a 10-day period was sufficient to restore these values.



## CONCLUSION

It is concluded that immobilization is able to induce alterations in the mechanical properties, reducing the muscle's ability to bear loads both in adult and in older animals.

Free remobilization did not demonstrate any effects in the short post-immobilization period in either age group, while remobilization by physical exercise presented a tendency for an increase in the LML, which was not sufficient to restore it to normal levels. We can conclude that the age or aging factor can interfere in a negative manner in the recovery response of the muscle

tissue with regards to the mechanical property of SML in the post-immobilization period.

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