MECHANICAL PROPERTIES OF GASTROCNEMIUS ELETROSTIMULATED AFTER IMMOBILIZATION

Leonardo César Carvalho, Juliana Cristina Polizello, Natalia Padula, Fernando Cassiolato Freitas, Antonio Carlos Shimano, Ana Claudia Mattiello-Sverzut

ABSTRACT

Introduction: Mechanical properties (MP) are clinically applicable tools for healthcare professionals working on the musculoskeletal system. Objectives: The aim of this study was to evaluate two protocols of neuromuscular electric stimulation (NMES) to improve MP regeneration of the myotendinous complex after segment immobilization in female rats. Materials and Methods: Fifty animals were equally distributed into five groups: Control (CG, n=10); Immobilized (IG, n=10); Immobilized and freely remobilized (IFG, n=10); Immobilized and NMES once /day (IEG1, n=10); Immobilized and MNES twice/day (IEG2, n=10). Immobilization was kept for 14 days, and remobilization was subsequently released for 10 days. NMES was applied for 10 days, post-immobilization, every morning for 10 minutes to IEG1 and every morning and afternoon (total 20 minutes) to the IEG2 group. After these procedures, the gastrocnemius muscle was submitted to the mechanical traction assay to evaluate stiffness, resilience, load and stretching at maximum limit MPs. Results: Immobilization reduced the MP values concerning load and stiffness (p<0.05). Results for NMES applied twice a day were less satisfactory than the ones obtained with one application or in the remobilized group (p> 0.05). Conclusion: It is concluded that the gastrocnemius muscle became structurally better organized through a single NMES application and by remobilization.

Keywords: *Immobilization. Electrical stimulation. Muscle skeletal.*

INTRODUCTION

Immobilization by segment[1,2], physical activity[1], neuromuscular electric stimulation (NMES)[3], among other modalities of external stimuli[4] are able to promote changes on the mechanical properties of skeletal muscle, tendon and bones.

The mechanical properties of tissues show two distinct phases: the elastic one, reflecting tissue extensibility without triggering rupture, and the plastic one, where structural tissue failures are found. When a skeletal muscle is passively tensioned without triggering contraction, a measurable strength can be obtained. This behavior has been named muscle stiffness, elasticity, extensibility or passive muscle tonus. [5,6]

The musculotendinous complex presents distinct structural components responsible for supporting tensional loads avoiding potential ruptures. Tendon, fascia, fascicles, contractile and cytoarchitectural proteins represent a set of structures offering resistance to external strengths.[7] These strengths are able to change the format and size of a muscle, and are directly influenced by tissue temperature, amount and duration of employed strength.[4,7]

Some structures account for this behavior, among which extracellular matrix[5,6] and tinine[2] are outlined, being known to be responsible for viscoelastic resistance of the musculotendinous complex.

Stiffness property can unveil that the tissue is able to promote a subtle stretching and bear a heavy load. Matheus et al.[3] said that this property, when reducibly expressed may leave a muscle susceptible to injuries when submitted to tension.

Schleip et al.[8] considered the hypothesis that intramuscular connective tissue, particularly its most superficial layer, the perimysium, would be able to more intensively adapt to external mechanical stimuli to the point of increasing its thickness and, ultimately, through collagen synthesis by fibroblasts, increasing muscle stiffness.

Immobilization of a segment is used in the treatment of musculoskeletal injuries, and this is known to induce deleterious changes on the mass, extensibility, strength and musculature resistance, associated to augmentation of intramuscular connective tissue[9] as well as ligament changes and joint stiffness.[10] It is reported that, after 48 hours of immobilization, the muscle develops atrophy, and, after 7 days, its mass is reduced by 37%.[11,12] Willians et al.[11] demonstrated that, in association with a reduced longitudinal length of fibers, there is a significant serial reduction of sarcomeres, and augmentation of perimysial and

---

endomysial connective tissue. Other authors confirmed these data using complementary experimental models.[13]

In parallel to these findings, intramuscular connective tissue augmentation[8] and macromolecular disorganization of its components have been suggested to determine significant modifications of the mechanical properties of the muscle submitted to immobilization.[5]

Neuromuscular electric stimulation (NMES) is a resource proven to preserve the cross-sectional area of a muscle and the synthesis of proteins, thus minimizing immobilization-induced atrophy.[7,14,15] When applied during the immobilization period, it has been able to avoid connective tissue augmentation.[11] In situations like the previous one, this resource has promoted the maintenance of mechanical properties of stiffness, load and stretching at proportional limit.[3]

The evaluation of mechanical properties of tissues consists of a very useful tool for establishing clinical/surgical protocols and rehabilitation programs, because it provides relevant knowledge about the potential adaptations occurring in muscles, tendons, ligaments and bone as a consequence of external stimuli.

In parallel, the use of NMES associated to immobilization period has been reported by some studies, but the protocols employed are different from each other[3,11] and the results achieved are inconsistent. Therefore, this study assessed some mechanical properties of the gastrocnemius muscle of female rats submitted to plastered immobilization, being subsequently remobilized, once and twice a day, in different periods, by NMES, or submitted to free mobilization.

MATERIALS AND METHODS

Animals

Fifty young adult female Wistar rats (Rattus Norvegicus Albinus), with average body mass of $200\pm30g$, supplied by the Central Animal Lab of Ribeirão Preto Medical School, University of São Paulo (USP) have been used in this study.

Experimental groups

This study was approved by the Committee of Ethics in Animal Experimentation (CEUA) - University of São Paulo - Ribeirão Preto Campus (SP), under the protocol number 06.1.692.53.8.
The animals were divided into 5 groups (n=10): Control (CG) – the animals in this group received no intervention, being kept for 24 days in cages at the Animal Lab of the Bioengineering Laboratory, University of São Paulo, with water and food *ad libitum*; Immobilized (IG) – gastrocnemius muscles in this group were immobilized for 14 days at shortening position; Immobilized and freely mobilized (IFG) - the animals in this group were previously immobilized for 14 days and then released in their cages for 10 days with water and food *ad libitum*. Immobilized and NMES/once a day (IEG1) - this group was immobilized for 14 days and then submitted to NMES technique applied for 10 consecutive days, once a day, for 10 minutes; Immobilized and submitted to NMES twice a day (IEG2) - the animals were immobilized for 14 days and submitted to the same NMES protocol as group IEG1, but applied twice a day at different moments, one in the morning and the other in the afternoon, with a 6-hour interval between applications. No sample was lost during the execution of experimental groups. The mean body mass at baseline was $202,08\pm15,91g$, and $233,33\pm16,57$ at study completion. Animals' age ranged from 42 to 49 days. After the interventions, the animals were sacrificed by means of an excessive dose of ketamine hydrochloride (80mg/kg) and xylazine hydrochloride

(15mg/kg) intraperitoneally. Then, the gastrocnemius muscle was dissected sparing proximal femoral and distal calcaneal intersections, which allowed for fixation of the muscle at the universal longitudinal traction mechanical assay machine. The gastrocnemius muscle was kept into 0.9% sodium chloride (NaCl) solution at a temperature of 25° until assays were conducted.

Immobilization

The immobilization of animals' right posterior limb was provided after applying intramuscular anesthetics, with a plastered cast including pelvis, hip, knee at total extension and with the ankle at maximum plantar flexion, as previously described.[4] In this method, gastrocnemius and soleus muscles remained at shortening position, especially due to the positioning at maximum plantar flexion, which enables the approach between the origin and insertion of both muscles.

Neuromuscular electric stimulation (NMES)

A neuromuscular electric stimulation equipment Bioset® brand model *Physiotonus Four*, with generator units of low frequency, 2-phase, (depolarized), asymmetric and short pulses, applied under controlled frequency was used.

For delivering an electric stimulus to the muscle, two electrodes were employed. The first one, a dispersive with area of $6cm^2$, was fixated to the lumbar region, and the second pen-like one, named as active, with 0.5cm in diameter was applied over the motor point of the right gastrocnemius muscle.

A gel layer between electrodes and contact regions was applied in order to allow for a better contact between skin and electrodes, enabling an easier delivery of the electric stimulus.

The parameters employed for stimulating the animals were the following: $50Hz^2$ frequency, on-duty cycle of 8s, off-duty cycle of 22s, and approximately 1mA (resistance of 100Ω) of power, able to promote a sustainable and visible contraction of the muscle. Before the development of the electric stimulation technique, all animals were submitted to trichotomy of the lumbar and ventral portions of the right gastrocnemius muscle. Such measure was adopted in order to facilitate the irradiation of the electric current. During NMES procedure, the knee remained at an extended position and the ankle at plantar flexion, which left muscles in a shortening position.

During the NMES treatment period, the animals were previously anesthetized with 0.05 ml combined solution of ketamine hydrochloride (80mg/kg) and xylazine hydrochloride (15mg/kg), for each 100 grams of body mass, injected into the muscle. In the group submitted to electric stimulation once a day, NMES was applied for 10 consecutive days, for 10 minutes a day, with 20 contractions being produced, applied at the same period of the day, i.e., in the morning. When used twice a day, NMES was applied once in the morning, and once in the afternoon, with a 6-hour interval between procedures, totaling an application time of 20 minutes and a total number of 40 contractions. In both NMES protocols, fatigue was not noticed.

Traction assay

The traction assay on rats' gastrocnemius muscle was done using the universal assay machine EMIC® - model DL10000, equipped with a 50Kgf load cell, belonging to the Bioengineering Laboratory of the Ribeirão Preto Medical School - USP. A special tool was employed, which enabled the fixation of the femur and the animal's paw, sparing muscle origin and insertion. After fixating the muscle to the tools on the universal assay machine, the muscle was submitted to axial traction. The parameters adopted

for the assay were 0.30Kgf pre-load, assay speed of 10mm/min, load and stretching limits of 8.00Kgf and 25mm, respectively; the assays were conducted until muscles were ruptured, and followed the previously described protocol.[1,3]

By means of an interface with a microcomputer (with built-in Tesc® software), a graph load versus stretching was built for each assay. (Figure 1) The mechanical properties assessed in this study were the following: stiffness, load, and stretching at maximum limit. Stiffness, which corresponds to the tangent of the angle formed by the straight line obtained at the elastic phase of the muscle (represented as N/m), showing the correlation between load and stretching during the elastic phase. The load at maximum limit (LML) reflects the heaviest load recorded on the plastic phase before the muscle was fully ruptured, represented as Newtons (N). The stretching at maximum limit (SML), which shows the maximum ability of a muscle to deform during the plastic phase when a traction load is applied without a full rupture, is represented as meters (x10^{-3}m).
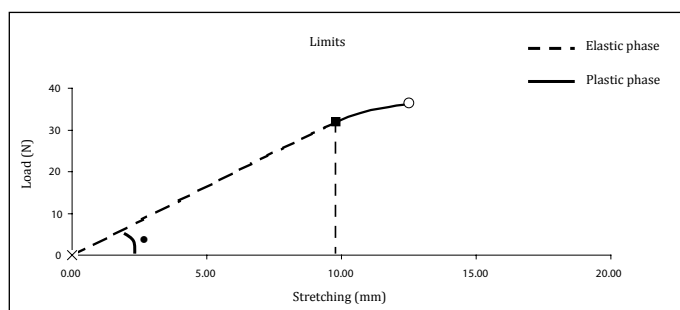

Figure 1 – *Load vs stretching curve obtained in the mechanical assays of the muscles. The ■ sign n represents the point concerning load and stretching at proportionality limit (PL) achieved at the elastic phase. The tangent of angle α represents values for stiffness. The triangle are represents the energy absorbed during the elastic phase (resilience). The sign ○ represents values for load and stretching at maximum limit (ML)*

Statistical analysis

Firstly, the results were tested for normality using the method by Kolmogorov and Smirnov. For comparison between groups, the ANOVA and Tukey-Kramer tests were used, with a significance level of 5%.

RESULTS

Immobilization significantly reduced the values for stiffness, load and stretching at maximum limit of gastrocnemius muscles compared to control group (p<0.05). (Table 1) Considering the properties of load and stiffness, the muscles of the animals submitted to immobilization showed lower values compared to those obtained with the application of NMES protocols (once and twice a day). (Table 1) Remobilization by NMES applied once and twice a day gave back a similar value for the mechanical property of load to the muscle, similar to control conditions (CG *vs* IEG1, CG *vs* IEG2 p>0.05), but the property of stretching at maximum limit was not reestablished. (Table 1)

When stiffness was assessed, NMES applied once a day (CG *vs* IEG1, p>0.05), as well as animals' release (CG *vs* IFG p>0.05) favored the reestablishment of this property, while NMES applied twice a day determined superior values to those obtained on control group (CG *vs* IEG2, p<0.05).

The mean and deviation values obtained with the assessment of the different properties for different experimental groups of the study are shown on Table 1.

Table 1 – *Mean and standard deviation values for mechanical properties of stiffness, load and stretching at maximum limit on the different study groups*

	LOAD (N)	STRETCHING (x 10^{-3}m)	STIFFNESS (x10^3 N/m)
GC	33.19 ± 1.66#	12.73 ± 1.19#,▲,●	3.62 ± 0.33#,●
GI	17.85 ± 5.71●,♦	9.96 ± 2.61♦	1.83 ± 0.71▲,●,♦
GIL	34.42 ± 5.55#	11.37 ± 1.63#,▲,●	3.43 ± 0.64#
GIE1	31.36 ± 4.50#	8.55 ± 1.45	4.24 ± 0.96#
GIE2	28.44 ± 3.59#	7.61 ± 1.28	4.74 ± 0.62#

■n: p<0.05 (compared to CG); #: p<0.05 (compared to IG); ▲ s: p<0.05 (compared to IEG1);● l: p<0.05 (compared to IEG2); ♦ ©; p<0.05 (compared to IFG).

DISCUSSION

The immobilization of a segment of the body is able to reduce mechanical properties of the musculotendinous complex.[1,3,5] In parallel, immobilization is known to change the direction of collagen fibers[5], promote atrophy of muscle fibers[13], reduce extensibility of sarcometric proteins (titin) and its isoforms (α and β)[2], as well as to promote changes on extracellular matrix[6] regarded as the potential causes for the reduced values of mechanical properties assessed in this study.

When a body segment is immobilized, muscles are subjected to adjustments, which may leave them susceptible to subsequent injuries by mechanical stress[5,9]; however, some of these changes can be avoided or even reversed with the use of remobilization techniques.[1] In our study, we adopted NMES and release of animals in their cages as remobilization methods.

The results achieved show that stiffness was considerably reduced in the immobilized group when compared to the other groups, corroborating with other researchers' findings.[1,3,5] When increased, the property in question reveals that the muscle is able to stand heavy loads and show a subtle stretching. This change may be present in cases of muscle shortening and at sites affected by injuries, with subsequent scarring tissue formation, because both change collagen fibers configurations, reducing tissue's stretching ability.[5] In our study, we noticed that the groups submitted to NMES once and twice a day showed the highest values for stretching at maximum limit when compared to other groups.

Cyclic mechanical loads work as an important regulator for different muscle functions, including gene expression on extracellular matrix[16], growth factors expression[6,16], as well as the synthesis of proteins and cytokines of the matrix.[17] NMES generates tensional cyclic loads able to promote structural changes on the musculotendinous complex, thus contributing to the modification of mechanical properties.

In our study, the results achieved on the groups submitted to intervention with NMES may have experienced increased intramuscular collagen degradation. Authors demonstrate that the muscles with prevalence of fast-contraction fibers show an increase of type-2 metalloproteinases expression after highly intensive exercises.[18] Nevertheless, when the activity is stopped, collagen synthesis is reestablished within 1-3 days.[6] In groups were NMES was applied, no daily intervals were provided for applications, which could have enhanced collagen degradation.

We believe in the hypothesis that this fact has contributed to the reduction of the values of stiffness and stretching at maximum limit for the groups submitted to NMES as compared to the immobilized-freely released group, once the animals included in the latter remained free in their cages after an immobiliza-

tion period, which allowed them to practice some low-intensity physical activity (ambulation) able to reduce metalloproteases expression[18], reestablish collagen synthesis and the subsequent reorientation of its fibers[5], this changes probably have contributed to the reestablishment of stiffness properties, increased load support and ability to stretch at the maximum limit of the immobilized- freely remobilized group.

Studies demonstrate that the reestablishment of mechanical properties of the gastrocnemius muscle to control parameters is achieved by releasing animals in their cages for four weeks[1]; however, this finding was not consistent to the ones by Matheus et al.[3], who released the animals for 10 days. In our study, release lasted 14 days, a sufficient time interval for promoting reestablishment to control parameters for all assessed properties.

In the group where the NMES intervention occurred twice a day, we noticed that the stiffness property showed superior values compares to control group, a result that could be regarded as beneficial, as shown by other authors.[3] Nevertheless, we should notice that, even when presenting stronger stiffness, the muscles of that group could stand lighter loads and experienced a subtle stretching when compared to the other groups, suggesting a stronger tissue susceptibility to injuries.

Grounded on the findings described above, and on that remobilization by physical exercises after immobilization changes the amount and orientation of the connective tissue on the muscle[10], we believe on the hypothesis that, even when perimyseal connective tissue expression is increased after an immobilization period, electric stimulation could have enhanced, just like highly intensive physical exercises[18], metalloproteinases activity, reducing collagen proliferation on the muscle with resultant reduction of the ability to stand tensional loads.

Another hypothesis suggested in this study is concerned to animals' alertness. The group submitted to immobilization and subsequent release remained at the restraint sites and showed no change in terms of alertness. These sites enabled animals to ambulate within their physiological limits, which did not occur with groups submitted to NMES, since they have been anesthetized and subsequently submitted to electric stimulation. When applied on a muscle with intact innervation, NMES promotes the recruitment of motor units in a distinct way from physiological patterns, i.e., initial recruitment of units susceptible to fatigue and then of the most resistant ones.[19] An electric stimulus applied on a given segment of the body elicit action potentials in all axons of the nerve - autonomic, motor and sensorial.[20] Thus, a consistent contraction as the one performed in our study may have been aggressive, intensifying the process of micro-injuries.

CONCLUSIONS

Therefore, the results achieved with this experimental model showed that, when applied after immobilization, NMES should be progressively used, with time intervals between days. It is not recommended to apply this technique more frequently than once a day. Free mobilization may be encouraged, respecting subject's pain limits. These findings should be taken into account during a rehabilitation process and when selecting a therapeutic approach, so that potential muscle injuries resulting from overloads are prevented at the most.

ACKNOWLEDGEMENTS

REFERENCES

1. Carvalho CMM, Shimano AC, Volpon JB. Efeitos da imobilização e do exercício físico em algumas propriedades mecânicas do músculo esquelético. Rev Bras Eng Biomed. 2002;18:65-73.
2. Goto K, Okuyama R, Honda M, Uchida H, Akema T, Ohira Y, Yoshioka T. Profiles of connectin (titin) in atrophied soleus muscle induced by unloading of rats. J Appl Physiol. 2003;94:897-902.
3. Matheus JPC, Oliveira JGP, Gomide LB, Volpon JB, Shimano AC. Efeitos da estimulação elétrica neuromuscular durante a imobilização nas propriedades mecânicas do músculo esquelético. Rev Bras Med Esporte. 2007;13:1-5.
4. Caiozzo VJ. Plasticity of skeletal muscle phenotype: mechanical consequences. Muscle Nerve. 2002;26:740-68.
5. Järvinen TA, Jozsa L, Kannus P, Järvinen TL, Järvinen M. Organization and distribution of intramuscular connective tissue in normal and immobilized skeletal muscles. J Muscle Res Cell Motil. 2002;23:245-54.
6. Kjær M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. Physiol Rev. 2004;84:649-98.
7. Lieber RL, Leonard ME, Brown-Maupin CG. Effects of muscle contraction on the load-strain properties of frog aponeurosis and tendon. Cells Tissues Organs. 2000;166:48-54.
8. Schleip R, Naylor IL, Ursu D, Melzer W, Zorn A, Wilke H et al. Passive muscle stiffness may be influenced by active contractility of intramuscular connective tissue. Med Hypotheses. 2006;66:66-71.
9. Silva C, Guirro R, Polacow M, Cancelliero K, Durigan J. Rat hindlimb joint immobilization with acrylic resin orthoses. Braz J Med Biol Res. 2006;39:979-85.
10. Kannus PL, Jozsa M, Kvist M, Vieno T, Jarvinen TA, Natri A. Jarvinen M. Free mobilization and low-to high-intensity exercise in immobilization-induced muscle atrophy. J Appl Physiol. 1998;84:1418-24.
11. Williams PE, Catanese T, Lucey EG, Goldspink G. The importance of stretch and contractile activity in the prevention of connective tissue accumulation in muscle. J Anat. 1988;158:109-14.
12. Gamrin L, Bug HE, Essen P, Tesch PA, Hultman E, Garlick PJ et al. The effect of unloading on protein synthesis in human skeletal muscle. Acta Physiol Scand. 1998; 63:369-77.
13. Mattiello-Sverzut AC, Carvalho LC, Cornachione A, Nagashima M, Neder L, Shimano AC. Morphological effects of electrical stimulation and intermittent muscle stretch after immobilization in soleus muscle. Histol Histopathol. 2006;21:957-64.
14. Arvidsson I, Arvidsson H, Eriksson E, Jansson E. Prevention of quadriceps wasting after immobilization: an evaluation of the effect of electrical stimulation. Orthopedics. 1986;9:1519-27.
15. Gibson JN, Smith K, Rennie MJ. Prevention of disuse muscle atrophy by means of electrical stimulation: maintenance of protein synthesis. Lancet. 1988;7:767-70.
16. Yang G, Crawford RC, Wang H-C. Proliferation and collagen production of human patellar tendon fibroblasts in response to cyclic uniaxial stretching in serum-free conditions. J Biomech. 2004;37:1543-50.
17. Arnoczky SP, Tian T, Lavagnino M, Gardner K, Schuler P, Morse P. Activation of stress-activated protein kinase (SAPK) in tendon cells following cyclic strain: the effects of strain frequency, strain magnitude, and cytosolic calcium. J Orthop Res. 2002;20:947-52.
18. Carmeli E, Moas M, Lennon S, Powers SK. High intensity exercise increases expression of matrix metalloproteinases in fast skeletal muscle fibres. Exp Physiol. 2005; 90:613-9.
19. Avramidis K, Strike PW, Taylor PN, Swain ID. Effectiveness of electric stimulation of the vastus medialis muscle in the rehabilitation of patients after total knee arthroplasty. Arch Phys Med Rehabil. 2003; 84:1850-3.
20. Qin L, Appell HJ, Chan KM, Maffulli N. Electrical stimulation prevents immobilization atrophy in skeletal muscle of rabbits. Arch Phys Med Rehabil. 1997;78:512-7.