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Effects of Ultrasound and Stretching on Skeletal Muscle Contusion in Rats: Immunohistochemistry Analysis

Efectos de Ultrasonido y Estiramiento en la Contusión Muscular Esqueletal en Ratas: Análisis Inmunohistoquímico

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SUMMARY: The aim of this study was to evaluate the effects of stretching and therapeutic ultrasound (TUS) on desmin and laminin contents of rat muscle after contusion. Male Wistar rats (n = 35, 8–9 weeks of age, 271 ± 14g body weight) were divided into five groups: Control group (CG) (n= 03); Injured group (IG) (n= 8); Injured + ultrasound group (IUSG) (n= 8); Injured+stretching group (ISG) (n= 8); Injured +ultrasound + stretching group (IUSSG) (n= 8). The application of ultrasound started 72 hours after the contusion, using the 50 % pulsed mode, 0.5 W/cm^2, 5 min, once a day, for five consecutive days. Passive manual stretching was started on the tenth day after injury, with four repetitions of 30 s each and 30 s rest between repetitions, once a day, five times per week, for a total of ten applications. After 22 days, the rats were euthanazied and the gastrocnemius of both limbs removed for desmin and laminin immunohistochemistry morphometric measurement. Analysis was conducted using ANOVA one way post-hoc Tukey to parametric data and Kruskall-Wallis for non-parametric data. The IUSSG animals showed a larger area of desmin than ISG ($p<0.05$). It was found a decrease in laminin comparing IUSG to IG. However, laminin area was higher in ISG than all groups ($p<0.05$). UST isolated or in combination with stretching influenced gastrocnemius regeneration in different manners. While stretching applied isolated enhanced gastrocnemius regeneration noticed by the increase in laminin area, in combination with TUS strengthened the muscle healing rising desmin area.

KEY WORDS: Muscle stretching exercises; Ultrasonic therapy; Desmin; Laminin, rats.

INTRODUCTION

Ninety percent of sports injuries are muscular, and of these, 60 % are caused by direct trauma or contusion (Smith *et al*., 2008). This type of injury results in functional impairment due to alterations such as capillary rupture (Hayashi *et al*., 2012) or rupture of the sarcolemma and myofilaments (Järvinen *et al*., 2007). Different therapeutic intervention techniques are used to stimulate muscle repair to recover function as follow: cryotherapy (Schaser *et al*., 2007); therapeutic ultrasound (TUS) (Piedade *et al*., 2008); L.A.S.E.R. (Falcai *et al*., 2015); and exercise, including early mobilization, stretching, and strength training (Järvinen *et al*.).

Despite stretching exercises (Torres *et al*., 2012) and TUS (Järvinen *et al*.) are widely used the evidences are still poor about their efficacy to treat muscle contusion. Therapeutic ultrasound can promote tissue repair by enhancing cell proliferation and protein synthesis during skeletal muscle regeneration (Piedade *et al*.; Rantanen *et al*., 1999; Shu *et al*., 2012). Rantanen *et al*. reported that pulsed ultrasound improved the development of myogenic precursor cells, myogenic cells, and fibrillar collagen in injured muscle. However, optimal repair of striated muscle requires not only an interaction between regenerating muscle

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cells and the extracellular matrix, but also the neovascularization and adhesion of the myofibers to the extracellular matrix, indicating the importance of proteins involved in this mechanism such as desmin and laminins (Wilkin *et al*., 2004; Piedade *et al*.; Rantanen *et al*.; Shu *et al*.; Cação-Benedini *et al*., 2014; Gianello *et al*., 2016).

It has been reported that physiotherapy program was more effective when stretching exercises was added to reduce time to return to sport after skeletal muscle injury (Pas *et al*., 2015).

Experimental animal models contribute to understanding the effects of lengthening stimulus, such as stretching, in the cellular mechanisms of skeletal muscle healing after contusion (Hwang *et al*., 2006; de Macedo *et al*., 2014, 2016). The involvement of desmin and laminin in force transmission has been described in the literature (De Deyne, 2001). An increase in sarcomerogenesis and an antifibrotic effect after a stretching protocol of injured rat gastrocnemius has been reported (de Macedo *et al*., 2014, 2016). Although the proteins desmin and laminin involved in the sarcomerogenesis and muscle healing, have not yet been investigated (Yu & Thornell, 2002; Cação-Benedini *et al*.; Gianelo *et al*., 2016).

The markers desmin and laminin are involved in the cellular mechanisms of skeletal muscle healing and force transmission in response to TUS or stretching (Piedade *et al*.; Cação-Benedini *et al*.; Gianelo *et al*.). Nevertheless, the literature did not explain how desmin and laminin act after muscle contusion treated with a combination of TUS and stretching. Thus, our hypotheses were: 1) The TUS would increase desmin and laminin contributing to skeletal muscle healing; 2) The stretching might strengthen the impact that TUS could have on the skeletal muscle regeneration. Hence, the present study aimed to evaluate the effects of ultrasound combined or not with stretching on injured gastrocnemius of young rats, in desmin and laminin content through immunohistochemistry.

MATERIAL AND METHOD

Animals. After approval by the ethics committee in animal experimentation (CEEA) of Federal University of Paraná (Certificate Number 491/2010), 35 young (8–9 weeks old, 271±14 g weight) albino Wistar rats were selected. To determine the sample size, a minimum of six animals per experimental group was considered, as in a homogeneous population, such as laboratory animals, a minimum sample size of six represents a 16 % opportunity per animal to present a distinct event (Zar, 1998). To control for unseen circumstances or possible losses, two additional animals were added to each experimental group. The animals were divided into five groups, housed four per cage and maintained in standard plastic cages under controlled environmental conditions (luminosity: 12 h light/dark cycle), with free access to water and pelleted feed, in the vivarium of the Integrated Faculties of Brazil (Unibrasil). The project was carried out according to the international norms of ethics in animal experimentation.

The animals (n=35) were divided into five groups: a control group (CG, n=3); an injured group (IG, n=8); an injured and ultrasound group (IUSG, n=8); an injured and stretching group (ISG, n=8); and an injured and ultrasound and stretching group (IUSSG, n=8) (Fig. 1).

No samples were lost from any of the experimental groups. After completing the experimental protocols, on Day 22 the animals were anesthetized and the gastrocnemius muscle was removed from both limbs, and then the muscles were weighed, and processed for the morphometric and immunohistochemistry desmin and laminin analysis.

Muscle injury. The animals were anesthetized with an intraperitoneal injection of Ketamine (95 mg/kg) and Xylazine (12 mg/kg) and maintained in a prone position with the right hind limb manually immobilized and the knee extended. The contusion was produced on the mid belly of the right gastrocnemius muscle (RGM) as described by Minamoto *et al*. (2001). The leg was shaved, the location for the projectile to fall over the muscle was defined with a marker, and the ink refreshed daily to guarantee that the TUS applications were applied over the same area.

Therapeutic ultrasound protocol. The animal was positioned in a supine position with the anterior limbs immobilized by one physiotherapist. To apply the TUS by the other physiotherapist, the right hind limb was positioned with the knee extended and ankle dorsiflexed at a 90-degree angle. Ultrasound was applied over the belly of the medial part of the RGM, with the treatment area previously shaved and marked with a marker pen. The animal was not under anesthesia. The effective radiation area of the probe was 1 cm^2. The equipment used was the Sonopulse especial (Ibramed, Brazil), at a frequency of 1MHz, intensity of 0.5 W/cm^2 spatial average temporal peak (SATP) (Piedade *et al*.), a pulsed duration of 50 % duty cycle, which corresponded to 0.25 W/cm^2 spatial average temporal area (SATA), with an application time of 5 minutes and using a total energy of 75J. A commercially available gel was used as a coupling agent (São Paulo Institute, Brazil). The equipment calibration was guaranteed by the manufacturer. The treatment of IUSG and

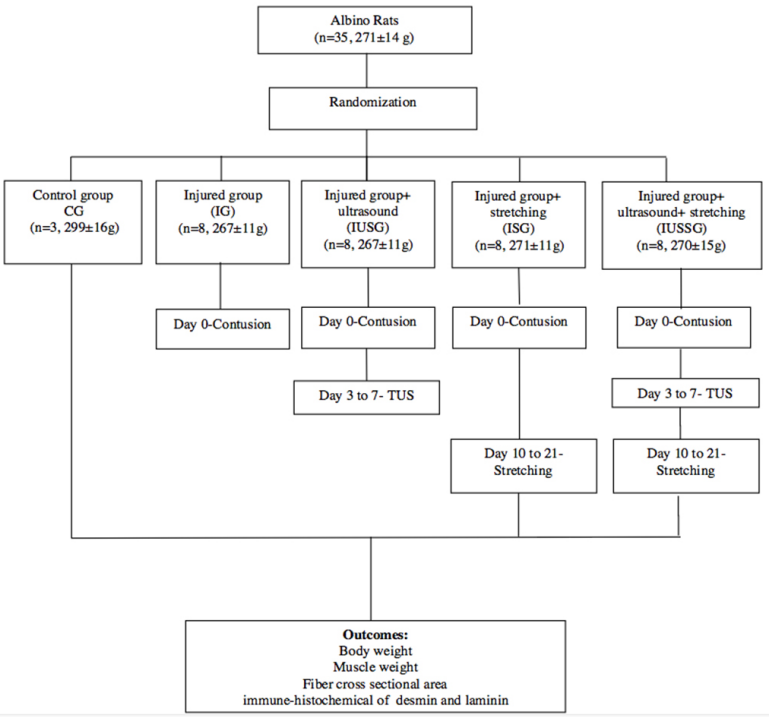

Fig. 1. Fluxograma of the study.

IUSSG consisted of, one application per day for five consecutive days, at the same time of day, starting at the 3rd day after contusion.

Gastrocnemius muscle stretching protocol. The animals were placed in the supine position to stretch the RGM, and the anterior limbs were immobilized by one physiotherapist while the other carried out the passive RGM manual stretching. The animal was not under anesthesia during this procedure. Maximum ankle dorsal flexion was carried out manually, with the knee extended, as described by Mattiello-Sverzut *et al*. (2006), considering the limit of the joint. The stretching protocol was started on Day 10 and maintained for 30 seconds per repetition (4 repetitions), with 30 seconds of rest between each repetition (Hwang *et al*.), once a day, five times per week from Monday to Friday, for two weeks, for a total of ten applications up to Day 21 of the experiment. The duration in seconds of each repetition and the rest periods were monitored using a stopwatch (Technos YP21518P, Technos, Brazil).

Chronological groups interventions. Pulsed ultrasound was applied during the inflammatory phase of the lesion, starting 72 hours after the contusion for 5 consecutive days (Plentz *et al*., 2008), considering the protocol described by Rantanen *et al*. The stretching protocol was applied between 10th to 21stdays, because 10 days after muscle injury, cells show high level of regeneration (Hwang *et al*.).

Euthanasia of the animals and removal of the muscles. The animals from all the groups were weighed and anesthetized with intraperitoneal injections of Ketamine (95 mg/kg) and Xylazine (12 mg/kg) to excise the gastrocnemius muscles. The muscles were periodically sprayed with a saline solution (0.9 % NaCl) to avoid drying out the tissue during dissection. Following the muscle excision, and while still under anesthesia, the animals were euthanized with an overdose of Ketamine and Xylazine anesthetics.

Each muscle was weighed separately using an analytical scale (Bioprecisa, Brazil) and divided at the middle into lateral and medial portions. The medial portion was then divided longitudinally into two parts, right and left, for analysis: The right portion was used to other analysis and the left part was fixed in a 10 % formalin solution for subsequent morphometric (muscle fiber cross sectional area) and immunohistochemistry analysis.

Fiber cross sectional area (FCSA). The cross-sectional areas of 100 muscle fibers were measured for each gastrocnemius muscle, chosen at random from the region of the muscle belly of the histological section, as described by Coutinho *et al*. (2004). The photomicrography was done through a light microscope (BX50-Olympus®, Brazil) (x20 objective) and the software Pro Plus 4.0 image was used to measure the muscle fiber cross-sectional area.

Immunohistochemistry. The medial part (left portion) of the belly of the gastrocnemius muscle was fixed in 10 % formalin for 72 hours and then embedded in paraffin. After fixing, 4 µm slices were cut from the block to make a new multi-sample block, also known as the Artisanal Tissue Microarray. These blocked samples were cut with a microtome (Leica RM 2145, Brazil) at 4 µm and affixed on the histological slides (76X26mm AutoWrite Green AdesinSakura, Brazil). After drying the slides overnight in an incubator at 60 ºC (Orion–model 502, Lynd, Wirral, England) and deparaffinizing, the antigen pre-treatment was started. The endogenous peroxidase (Streptavidin-Peroxidase, Brazil) was blocked with a hydrogen peroxide solution diluted in 5 % methanol. The slides were incubated in desmin (Dako, Brazil) and laminin (Dako, Brazil) antibody solution overnight at 4 ºC. The stain DAB (Diaminobenzidine, Brazil) (1:1) was used until the slide contents appeared brown in color, and then destained using Harris hematoxiline (Alpha Tec, Brazil). If positive, the desmin and laminin antibodies can stain intermediary filament and basal lamina brown, respectively.

The Image Pro Plus® program was used to read the laminin and desmin antibodies with the aid of a Dino-eye® camera and an optical microscope (BX50-Olympus®, Brazil).

A photomicrograph of a positive control slide was made using a magnification of 400x. A sample of the brown coloration for the program was used as a positive control to allow the quantification of desmin and laminin by the colorimetric method. This photomicrograph was considered the "mask" (control slide) as reported by Calvi *et al*. (2012), made from a specific structure demarcated into an image. After selecting a specific structure, the program identified the structure's pixels, and then the software selected adjacent pixels with similar color.

A field 200 x 200 μm^2 in size containing only muscle fibers was selected from the histological slide using the Image Pro Plus program to quantify the areas of the desmin and laminin proteins. The "mask" was superimposed on each histological slide photographed, and the program automatically read the totality of the immune-positive brown color area previously standardized in the field photographed, thereby quantifying the desmin or laminin areas.

Initially ten photomicrographs were taken of the right gastrocnemius muscle of each sample in each block, that is, from each rat in each group. The five images containing the fewest artifacts and greatest number of fibers were selected. Artifacts included the following: reagent deposits, dust, bent fibers, torn fibers, or badly focused fibers. After reading the five selected images, the arithmetic mean of the measured area from each slide was calculated. The program automatically read the totality of the immune-positive area in μm^2 identifying the brown color previously standardized.

Statistical Analysis. The Shapiro-Wilks and Levene tests were carried out to evaluate the normality and homogeneity of data distribution, respectively ($p>0.05$). The data were considered non-parametric if assumptions of normality and/or homogeneity of variance were not met.

The between groups comparisons were carried out through one-way ANOVA and post-hoc Tukey tests for the parametric data and Kruskal-Wallis tests for the non-parametric data, considering a 5 % significance level ($p< 0.05$, two-tailed). Parametric and non-parametric data are expressed as the mean standard deviation and median, minimum, maximum. Statistical analysis was performed using SPSS 25.0.

RESULTS

There was an increase in body weight in the IUSG and IG animals compared to the ISG and IUSSG, also in the CG animals in comparison to the ISG and IUSSG animals (ANOVA, $p< 0.05$). The gastrocnemius muscle of the IUSG animals was heavier than that of the ISG. The IUSSG relative muscle wet weight-to-body weight was greater than IG animals (Kruskall-Wallis, $p< 0.05$). The IG samples cross-sectional area (FCSA) was larger than the ISG samples (12787±995 μm^2 vs 8721±2341 μm^2, ANOVA, $p = 0.01$). The data are described in Table I.

The area of desmin content in the IUSSG samples was larger than that found in the ISG samples (Kruskal-Wallis, $p< 0.02$, Table II). Figure 2 shows the desmin (brown color) more pronounced in the IUSSG samples.

The results obtained for the laminin area in the muscle showed normal distribution and homogeneity (Shapiro-Wilks and levene respectively, $p>0.05$). It was found a decrease in laminin comparing IUSG to IG. Nevertheless, laminin area in ISG was higher than all the groups (ANOVA, $p<0.05$). The data are described in Table II. Figure 3 shows the laminin (brown color) more pronounced in the ISG samples.

Table I. Body weight, gastrocnemius muscle weight, relative muscle wet weight-to-body-weight and FCSA in all experimental groups.

Group	Final Body weight (g)	Gastrocnemius wet weight (g)	MW/BW (mg/g)	FCSA (μm^2)
IG	350.0±17.9*	1.7±0.1	5.0	12787±995¥
IUSG	352.2±19.1*	1.8±0.2#	4.9	10819±2612
ISG	323.±17.6§	1.5±0.3	4.9	8721±2341
IUSSG	317.7±15.8§	1.7±0.1	5.6†	10300±924
CG	383.3±24.5	1.8±0.1	4.7	13814±2904

IG, injured group; IUSG, injured+ultrasound group; ISG, injured+stretching group; IUSSG, injured+ultrasound+stretching group; CG, control group; MW, muscle weight; BW, body weight; FCSA, fiber cross sectional area. All data are expressed as the mean±standard error mean.*compared to ISG and IUSSG ($p<0.05$); §compared to CG ($p<0.05$); #compared to ISG; †compared to IG ($p<0.05$); ¥ compared to ISG ($p<0.05$).

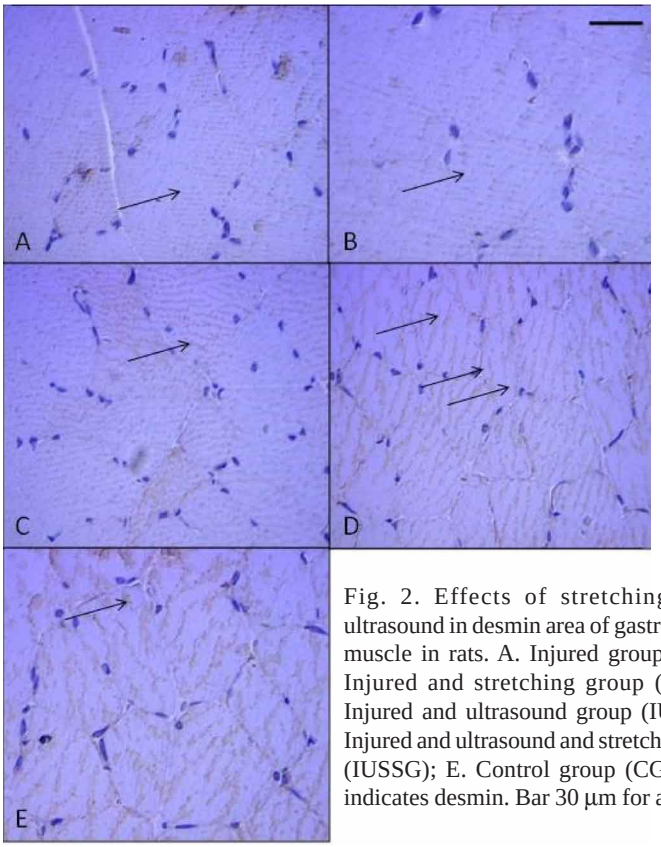

Fig. 2. Effects of stretching and/or ultrasound in desmin area of gastrocnemius muscle in rats. A. Injured group (IG); B. Injured and stretching group (ISG); C. Injured and ultrasound group (IUSG); D. Injured and ultrasound and stretching group (IUSSG); E. Control group (CG). Arrow indicates desmin. Bar 30 μm for all panels.

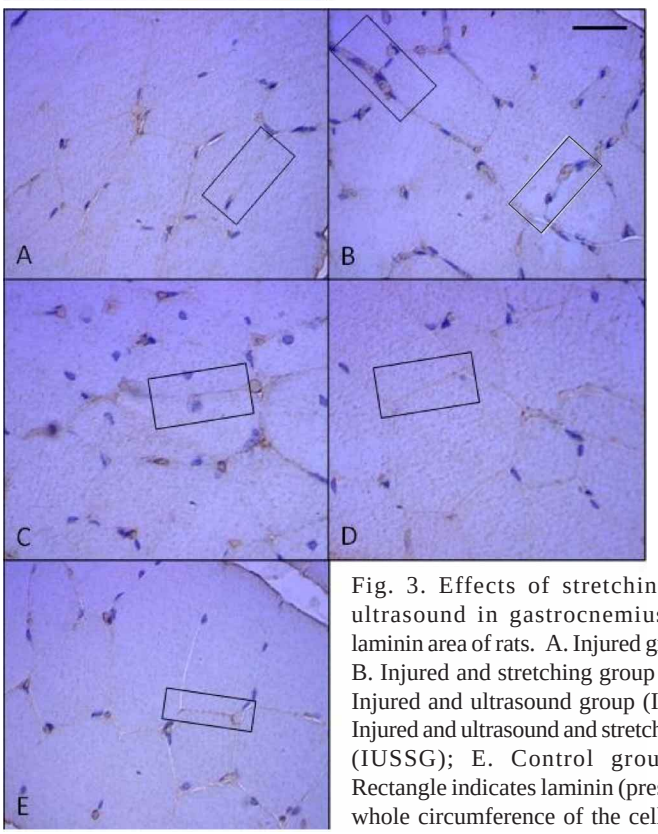

Fig. 3. Effects of stretching and/or ultrasound in gastrocnemius muscle laminin area of rats. A. Injured group (IG); B. Injured and stretching group (ISG); C. Injured and ultrasound group (IUSG); D. Injured and ultrasound and stretching group (IUSSG); E. Control group (CG). Rectangle indicates laminin (present in the whole circumference of the cell). Bar 30 μm for all panels.

DISCUSSION

The stretching applied isolated enhanced gastrocnemius regeneration indicated by the increase in lamina area. The combination of TUS and stretching strengthened the muscle healing as shown in the rise of desmin area.

Differences in body weight among experimental groups were noticed, in particular, in the stretched muscle. For instance, the animals subjected to stretching intervention gained approximately 17–19 % body weight compared to the injury or ultrasound groups, which gained approximately 31–32 % body weight. Compared to the final body weight among the injury groups, a decrease of approximately 8–10 % was found in the animals receiving the stretching therapy compared to those in the injury or ultrasound groups. As the CG animals had the highest final body weight, suggesting that exposure to a range of stimuli, including contusion, ultrasound, and stretching, may have negatively impacted the absolute body weight gain.

Regarding FCSA it was found that the IG was larger than that in the ISG muscle, although there was no difference with the control group. The FCSA difference may have been influenced by the increase in body weight, although no increase in muscle weight was detected. In this study, ultrasound and stretching produced no changes in FCSA. Markert *et al.* (2005) evaluated the effects of TUS and low-intensity walking exercise for 20 minutes on treadmill at a velocity of 14 m/min for 4 days, starting 24 hours after mechanical injury of the gastrocnemius, and found no increase in FCSA. As IG muscle had a greater FCSA than ISG muscle, stretching may have interfered with edema reduction and regeneration, but was not sufficient to stimulate radial or transverse muscle growth, as reported in other studies (Rantanen *et al.*; Market *et al.*, 2005).

In the present study, the injured gastrocnemius muscle treated by both ultrasound and stretching showed a larger area of desmin than by stretching isolated or just ultrasound. Other studies found an increase in immunereactivity of the desmin, and in the number of myoblasts and myotubes when muscle submitted

Table II. Effects of stretching and/or therapeutic ultrasound in the desmin and laminin area of rat right gastrocnemius muscle.

	Desmin area (μm_)	Laminin area (μm_)
Group	Mean±SD (median, minimum, maximum)	Mean±SD (median, minimum, maximum)
IG	1746.1±766.7 (1835.7, 802.8, 3154.9)	279.6±76.0 (217.7, 187.4, 378.1)
IUSG	1858.9±781.0 (1686.5, 908.0, 3428.2)	163.4±27.1 (163.3, 115.4, 214.6)§
ISG	1549.5±513.2 (1481.7, 1019.6, 2679.2)	498.5±81.7 (473.5, 362.3, 594.6)#
IUSSG	2960.7±1113.7 (3332.8, 1521.8, 4593.0)*	223.9±39.7 (220.1, 155.2, 276.1)
CG	2585.8±924.5 (2081.9, 1775.8, 3593.0)	216.2±39.0 (199.0, 173.7, 250.6)

IG, injured group; IUSG, injured+ultrasound group; ISG, injured+stretching group; IUSSG, injured+ultrasound+stretching group; CG, control group; SD, standard deviation. All data were expressed as the mean±standard deviation (median; minimum-maximum). *p<0.05 compared to ISG; #p<0.05 compared to IG, IUSG, IUSSG and CG; §compared to IG.

to contusion was treated with ultrasound (Rantanen *et al*.; Hwang *et al*.; Plentz *et al*.; Piedade *et al*.).

It has been reported that pulsed TUS (3MHz, 20 % duty cycle, 1.5W/cm^2), applied six hours and three days after muscle contusion until tenth day, increased the number of myogenic cell precursors and the proliferation of fibroblasts without increasing the formation of myotubes, as evaluated by desmin level (Rantanen *et al*.). In the present study, no increase in desmin was observed in the IUSG muscle, probably because TUS was started 3 days after the contusion and applied during five days, while the cited authors started 6 hours later and applied during 10 days. It could be hypothesized that as early as possible should be initiated the UST.

It was investigated the effects of different doses (0.25, 0.5, and 0.75 W/cm^2; 15, 30, and 45 J, respectively) of pulsed (20 %) ultrasound at 3MHz, applied 24 hours after mechanical muscle contusion in rats, evaluated based on the amounts of desmin up to Days 4, 7, and 14 (Shu *et al*.). The study revealed a significant increase in desmin in all groups compared to the control group ($p< 0.005$). In addition, the expression of desmin increased from Day 4 to Day 14, with the peak value on Day 7, but showed no significant difference between the groups (Shu *et al*.). Our study used a deeper frequency of 1MHz, and 75 J of energy for fewer days, possibly suggesting insufficient stimulus for desmin production.

One study, which assessed the effects of pulsed ultrasound (1MHz, 50 %, 0.57W/cm^2, 5min) applied after 48 hours of lacerated gastrocnemius muscles of rats, found larger amounts of myoblasts and myotubes on Day 14, analyzed by the amounts of positive-desmin in the regeneration zone. The authors associated the increase in desmin-positive myoblasts and myotubes to the proliferation and differentiation of satellite cells induced by TUS, indicating enhanced muscle regeneration (Piedade *et al*.).

In the present study, no statistically significant difference was found in the area of desmin in injured muscle subjected only to ultrasound (1 MHz, 50 % duty cycle, 0.5 W/cm^2). This result could be attributed to the fact that the rats were treated with TUS up to Day 8 and the desmin evaluated on Day 22. Thus, the stimulus may have been insufficient to activate the satellite cells to differentiate into myoblasts, and subsequently to mark desmin.

The studies cited earlier evaluated the isolated effects of ultrasound, without associating it with other therapeutic equipments or exercise. However, the present study found a smaller area of desmin in the group treated by stretching only when compared with the group treated with both ultrasound and stretching. It may be hypothesized that an isolated application—either ultrasound or stretching—did not generate sufficient stimulus to modify the regeneration process evaluated by desmin levels. Another hypothesis may be that the applied stretching protocol impaired the regeneration process, either by the use of maximum passive dorsiflexion or by the total duration maintained.

Starting the stretching treatment in the present study on Day 10 after the contusion may have been too early. The best results with respect to the level of fibrosis (50 % less), the number of myofibrils regenerated, and greater muscle length were observed when the stretching was started 14 days after the contusion (Hwang *et al*.). However, even starting the stretching ten days after the contusion, our previously results (de Macedo *et al*., 2014) showed sarcomerogenesis and antifibrotic effects.

When stretching was associated with TUS, as in IUSSG animals, a larger area of desmin was detected which may be attributed to the longer, three-week duration of the stimulus in therapeutic combination. The IUSSG animals were the only group that received intervention every day, first with ultrasound, and then with stretching.

Satellite cells are quiescent in healthy muscle fiber, and in the event of injury they quickly start to divide and replace the injured tissue (Lieber, 2002). There is considerable evidence demonstrating that satellite cells represent a

population of stem cells that can differentiate into muscle cells or other types of cell (Lieber). When activated, satellite cells migrate to the injured region, proliferate and differentiate into myoblasts, and then unite to form myotubes. Studies have shown that both ultrasound (Lieber; Hwang *et al*.) and stretching, by way of its mechanical effect (Tatsumi *et al*., 2001), stimulate satellite cells.

Thus it can be proposed that the increase in desmin resulting from the association of TUS with stretching may have enhanced the muscle regeneration process by activating the satellite cells. In the present study the satellite cells were not marked, although we noticed centralized nucleus in the histological analysis, data not shown in this manuscript, but it could indicate its activation. The present authors suggest marking the satellite cells in a future study to reveal the effects of TUS and stretching on the muscle regeneration mechanism.

The present study showed that ISG groups present higher laminin amount area than other groups. When a muscle is stretched, it transmits the force to a set of structures located in the extra milieu, such as laminin (Cação-Benedini *et al*.). Laminins play important roles in tissue structure and maintenance, cell signaling, adhesion, migration and it is important for the success of regeneration, for the formation and spatial orientation of the new myotubes, and for minimizing the development of fibrosis (Durbeej, 2010). The laminins can contribute to tensile strength in adults, and serve as a scaffold to orient regenerating myotubes after muscle damage. Stretching is a technique capable of inducing myofibrillogenesis, by lengthening longitudinally the costameric structures, intermediate filaments, contractile proteins and extracellular matrix elements (Coutinho *et al*.). De Deyne reported that passive stretch is transmitted from the endomysium (extracellular matrix), across the muscle fiber membrane (sarcolemma), to noncontractile elements (costamere, like desmin) to the Z. line (laminin, fibronectin) have the capacity to bind integral membrane proteins (integrins, dystroglycan complex). Although our study found an increase in laminin area in the group treated with stretching, it was not possible to observe the same response in the group that associated ultrasound and stretching or that applied only ultrasound. Rantanen *et al*. study showed that ultrasound after muscle injury promoted the satellite cell proliferation phase of the myoregeneration but not affected myotube production inside the basal lamina, market by laminin. Therefore it is possible that the previous application of ultrasound and its mechanical effect is not sufficient to increase the production of laminin.

Our study was limited by using a general immunohistochemistry technique for laminin and not using laminin a2, specific for skeletal muscle. Further studies should investigate the effects of stretching in the acute phase of muscle injury, and also evaluate other glycoproteins such as integrins, collagen IV, and fibronectin, and use molecular biology analysis to demonstrate the mechanisms involved in the skeletal muscle regeneration process.

The stretching effect may complement TUS as reported by other studies which have shown that TUS applied to injured muscle tissue displayed a better structural arrangement with a more regular alignment of collagen muscle fibers, and that stretching induced antifibrotic and regenerative effects (Market *et al*., 2005; Hwang *et al*.; Piedade *et al*.). The present study has demonstrated larger areas of desmin in IUSSG tissue when compared to ISG tissue and larger laminin area in ISG than other groups suggesting enhanced skeletal muscle regeneration. However, better treatment evidence would exist if a statistical difference had been found between the control or injured groups and treatments in desmin area. The present data are insufficient to indicate pulsed TUS application to muscle lesions without restriction, as the optimal repair of striated muscle requires not only morphologic investigation, but functional tests which were not conducted. Stretching can be used after 10 days of contusion for promoting muscle healing.

CONCLUSIONS

The results of our study indicated an improvement in the muscle regeneration in different ways. When both ultrasound and stretching therapies were applied reinforced the muscle healing by increasing desmin area. Although stretching applied isolated improved gastrocnemius regeneration detected by rising laminin area.

DE MACEDO, A. C. B.; YWAZAKI, J. L.; MARTINS, A. P. C.; AZEVEDO, M. L. V.; NORONHA, L. & GOMES, A. R. S. Efectos del ultrasonido y estiramiento en la contusión muscular esqueletal en ratas: Análisis inmunohistoquímico. *Int. J. Morphol., 38(5)*:1288-1295, 2020.

RESUMEN: El objetivo de este estudio fue evaluar los efectos del estiramiento y la ecografía en los contenidos de desmina y laminina del músculo de rata después de la lesión. Ratas Wistar macho (n = 35, 8–9 semanas de edad, 271 ± 14 g de peso corporal) se dividieron en cinco grupos: grupo de control (CG) (n = 03); Grupo lesionado (GL) (n = 8); Lesionado + grupo de ultrasonido (LGU) (n = 8); Lesionado +grupo de estiramiento (LGE) (n = 8); Lesionado + ultrasonido + grupo de estiramiento (LUGE) (n = 8). La aplicación de ultrasonido comenzó 72 horas después de la lesión, usando el modo pulsado al 50 %, 0,5W / cm^2, 5 min, una vez al día, durante cinco días consecutivos. El estiramiento manual pasivo se inició el décimo

día después de la lesión, con cuatro repeticiones de 30 seg cada una y 30 seg de descanso entre repeticiones, una vez al día, cinco veces por semana, para un total de diez aplicaciones. Las ratas fueron sacrificadas después de 22 días, y se extrajo el músculo gastrocnemio de ambos miembros para la medición morfométrica de desmina y laminina a través de inmunohistoquímica. El análisis se realizó utilizando ANOVA unidireccional Tukey post-hoc para datos paramétricos y Kruskall-Wallis para datos no paramétricos. Los animales LUGE mostraron un área mayor de desmina que LGE ($p < 0,05$). Se encontró una disminución en la laminina comparando LGU con GL. Sin embargo, el área de laminina fue mayor en LGE que en todos los grupos ($p < 0,05$). El tratamiento con ultrasonido aislado o en combinación con estiramiento influyó en la regeneración del músculo gastrocnemio de diferentes maneras. Si bien el estiramiento aplicado, en combinación con tratamiento de ultrasonido, fortaleció el área de desmina, la regeneración del músculo gastrocnemio mejoró por el aumento en el área de laminina aumentando la curación muscular.

PALABRAS CLAVE: Ejercicios de estiramiento muscular; Terapia ultrasónica; Desmina; Laminina; Ratas.

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