Abstract

Introduction

Atrophy and muscle shortening due to articular immobilisation are common problems in musculoskeletal rehabilitation. Muscle stretching mechanical stimuli might be considered as the golden standard procedure to improve muscle flexibility in rehabilitation. Muscle stretching generates mechanotransduction, potentiating specific gene expression and promotes sarcomerogenesis and extracellular matrix remodelling on shortened and atrophied muscles.

Hypothesis

Diacutaneous fibrolysis, like stretching, uses an external force to stress connective and muscle tissues mechanically to treat muscle shortening; thus, it is widely used in clinical practice even if there is no evidence to support it. Considering this subject, we have hypothesised that diacutaneous fibrolysis can generate mechanotransduction, affecting muscle hypertrophy and extracellular matrix remodelling after immobilisation.

Evaluation of hypothesis

We have designed a laboratory experimental study with a sample of 50 rats. The sample was randomly divided into five groups: Control group (n = 10) with non-immobilised rats; 3-week immobilisation group (n = 10); 3-week immobilisation/3-week non-immobilisation group (n = 10); 3-week immobilisation/3-week stretching group (n = 10); and 3-week immobilisation/3-week diacutaneous fibrolysis group (n = 10). All rats had their left tibiotarsal joint immobilised in maximum plantar flexion with the orthotics for 3 consecutive weeks. After the immobilisation period, the intervention groups received their respective intervention on their left triceps suralis for 3 weeks. Dependent variables of the study were sarcomere analysis, polymerase chain reaction, connective tissue density, collagen birefringence and matrix metalloproteinases. Statistical analysis was performed using analysis of variance and Duncan post hoc test was applied for differences between groups. For all calculations, a 5% (p < 0.05) significance level was established.

Conclusion

If the hypothesis is confirmed, the present study might provide evidence to support the use of this physical therapy resource widely used to treat muscle dysfunctions.

Introduction

Muscle plasticity is a remarkable mechanical property: it is the ability of muscle cells to alter their structure and function in accordance to different stimuli. Articular immobilisation leads to muscle atrophy and rigidity, characterised by decrease in muscle fibre protein content and size1 and increase in their connective tissue2.

Data show that during the first 6 hours of articular immobilisation, the synthesis of muscle protein is reduced, and within 72 hours, it might reduce the muscle mass up to 30% of its original size4,14. Seven days of immobilisation results in loss of muscle fibres, reduces the number of serial and parallel sarcomeres and leads to muscle atrophy and shortening6,8. Evidence shows that the increase in connective tissue diminishes blood flow, water and proteoglycans into muscle fibres; binding of abnormal collagen fibres occurs, which induces rigidity and loss of flexibility of the connective tissue7,9.

Passive muscle stretching is considered as an effective resource and is often used to increase muscle and joint flexibility. Previous studies have showed that muscle stretching promotes sarcomerogenesis (contractile protein synthesis triggered by specific muscle gene potentiation)9–12 by mechanotransduction (mechanical stimulus conversion into chemical activity)13–15. During muscle stretching, mechanical stimuli are first transmitted to the extracellular matrix (ECM), and the integrins on their membrane detect those stimuli and transmit them to the cell interior; activating a series of nuclear proteins responsible for modifying the specific gene transcription that regulates sarcomerogenesis16.

Stretching–induced mechanotransduction affects connective tissue remodelling through matrix metalloproteinases (MMPs) because they degrade ECM components. MMPs play a role in both tissue function and development, which include pathological processes. Coutinho et al.2.
Demonstrated that daily sessions of muscle stretching for 3 weeks were enough to rearrange collagen bands of rats’ immobilised muscle, showing positive outcomes of muscle stretching in ECM remodelling.

Diacutaneous fibrolysis17,18 is a non–invasive physiotherapeutic method to treat musculoskeletal disorders and movement restriction19 that uses a stainless steel hook to generate mechanical stimulus20 from lateral traction movement of the muscle belly. This traction technique is performed manually with smooth and precise anatomical knowledge. Despite the extensive use of diacutaneous fibrolysis in physical therapy clinical practice to release adherences between muscle, aponeurosis and tendons21, we found no evidence of its effects on muscle plasticity.

**Hypothesis**

To the best of our knowledge, no previous study has described diacutaneous fibrolysis in skeletal muscle adaptations. But, Veszely et al.22 analysed the effects of diacutaneous fibrolysis on human skeletal muscles through the use of surface electromyography, confirming that the traction technique presented neural and mechanical effects similar to those observed in studies which assessed passive muscle stretching. We obtained the significant T\text{max}/M\text{max} (mV) reflex reduction and passive mechanical tension (N\text{max}) of the triceps suralis (TS) muscle of young adults 30 minutes after diacutaneous fibrolysis.

Diacutaneous fibrolysis is an external force that stimulates muscle and connective tissues mechanically. This hypothesis is based on the affirmative that the traction technique produces enough muscle tension to provoke mechanotransduction. Thus, we hypothesise that the diacutaneous fibrolysis traction technique affects muscle plasticity, promoting sarcomerogenesis and ECM remodelling in muscle atrophy and shortening, based on the work of Veszely et al.22.

Figure 1 shows the traction technique on the muscle belly of the biceps braquialis of a patient presenting muscle shortening, which shows that the greater curve of the hook fits the muscle surface involving it. Figure 2 shows the traction technique adapted to rats.

Therefore, the objective is to assess diacutaneous fibrolysis effects on muscle atrophy signalling pathways, sarcomerogenesis and ECM remodelling of muscles in disuse and compare them to the effects of passive muscle stretching by applying the same analysis.

**Evaluation of Hypothesis**

The authors have referenced some of their own studies in this hypothesis. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed. Animal care was in accordance with the institution guidelines.

The authors have designed an experimental research because of the research problem. The research was conducted in accordance with the Guide for Care and Use of Laboratory Animals of the University of Brasilia.

A sample of 50 rats (Wistar lineage *Rattus norvegicus*) was selected from the University of Brasilia’s vivarium. These animals were randomly divided into five groups: Control group (CG, n = 10) with non–immobilised rats; 3–week immobilisation group (IG3, n = 10); 3–week immobilisation/3–week non–immobilisation group (INIG3, n = 10); 3–week immobilisation/3–week stretching group (STR, n = 10); and 3–week immobilisation/3–week diacutaneous fibrolysis group (DF, n = 10). All rats had their left tibiotarsal joint immobilised in maximum plantar flexion with the orthotics described by Coutinho et al.23 for 3 consecutive weeks. After the immobilisation period, STR and DF groups received their respective intervention on their left TS for 3 weeks.

The STR group had the TS passively stretched by maintaining a maximum tibiotarsal dorsiflexion for 1 minute in accordance with the

**Figure 1:** Traction technique on the muscle belly of the biceps braquialis of a patient presenting muscle shortening.

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protocol of Coutinho et al. Each rat was subjected to 3 sessions/day with 1 minute interval between each session, and 5 days/week. The authors chose manual passive stretching because it is most commonly used in humans. The DF group received the traction technique at the left TS with the tibiotarsal joint in the neutral position, and the traction was performed with the hook's greater curve, following the protocol: (1) 20 traction movements of the medial portion of the muscle belly; (2) 20 traction movements of the lateral portion. The hook always fits the muscle surface with slow and smooth movements.

The INIG3 rats had their tibiotarsal joints immobilised for 3 weeks and after the joint release, they walked freely inside the cage for 3 weeks, with no intervention. The IG3 group was subjected to euthanasia after orthotics removal; and the CG group was also subjected to the same procedure after 3 weeks of free walk inside the cage.

All animals subjected to immobilisation and/or intervention received an intraperitoneal injection of Xylazine (12 mg/kg) and Ketamine (95 mg/kg) for anaesthesia. All were euthanised with anaesthetic overdose. Then, the soleus muscle was removed and fixated in 5% paraformaldehyde aqueous solution for 4 hours at ambient temperature. Then, they were dehydrated by sequential immersion in alcoholic solutions with a decreasing water percentage, diaphonised in Xylol, and embedded in hot paraffin. The paraffin blocks were refrigerated until the microscopic section. For every 250-μm slice, a sample of 5-μm thickness was selected for further analysis. The morphometric study was conducted by only one observer using an optical microscope with topographic magnification and the images were captured by AxioCam (Carl Zeiss, Germany) microscope camera. To isolate muscle fibres, the samples were fixated in polystyrene plates, immersed in 2.5% glutaraldehyde solution for 3 hours and in 30% nitric acid solution for 2 days for connective tissue hydrolysis; after that, they were stored in 50% glycerol solution.

Dependent variables
Sarcomere analysis
We used a stereomicroscope (Stemi DRC, Zeiss) to isolate muscle fibres in a Petri dish with forceps. Histological sections were stained with Gomori’s trichrome and were analysed by visible light optical microscopy with an immersion lens (100×). Five muscle fibres from the medial portion were selected from all the slides and sarcomere count was started, dividing the slides into six distinct fields of 50 μm, with a total of 300 μm/muscle fibres. The number of serial sarcomeres along the fibre were estimated by a simple rule of three.

Polymerase chain reaction
The fragments were readily stored at −80°C after dissection for further total RNA extraction. The fragments were homogenised with Trizol (1 ml), according to the manufacturer’s protocol (Life Technologies Inc., USA). Extracted RNAs were dissolved in Tris–HCL and in ethylenediamine tetra-acetic acid (EDTA) (pH 7.6), and quantified by spectrophotometry. RNA reverse transcription was conducted in a thermocycler (Eppendorf AG, Germany), using reaction mixture consisting of 1 μg of total RNA, 4 μl of 5× Buffer, 0.8 mM dNTPs (Invitrogen), 4 mM DTT (Invitrogen), 200 μl of reverse transcriptase (Invitrogen).

Primer pairs were coded for the myostatin (GenBank, AF019624), atrogin–1 (GenBank, AF441120), and the transcription factor IID (TFIID; GenBank, D01034) using Primer Express software v2.0 (Applied Biosystems, Foster City, Califórnia). TFIID were used as an internal control because of the constant expression levels in musculoskeletal stretching models. We obtained MyoD muscle-specific transcription from Hill et al. and primers were synthesised by Imprint kit (Sigma–Aldrich Co. LLC.). RNA transcription levels for experimental and control groups were simultaneously analysed in duplicates.

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Diacutaneous fibrolysis might be more beneficial than passive stretching in clinical practice due to two aspects: (i) the procedure does not produce pain or discomfort; and (ii) does not promote joint movement. These aspects might be relevant for muscle stimulation in early treatment of intra-articular fractures, luxation and muscle lesions, when joint movement is counter-indicative.

Conclusion
If the hypothesis is confirmed, the present study might provide evidence to support the use of this physical therapy resource widely used to treat muscle dysfunctions.

Abbreviations list
ANOVA, analysis of variance; GG, Control group; DF, diacutaneous fibrolysis; ECM, extracellular matrix; EDTA, ethylenediamine tetra-acetic acid; IG, immobilisation group; INIG, 3-week immobilisation/3-week non-immobilisation group; MMP, matrix metalloproteinases; STR, stretching group; TFIID, transcription factor IID; TS, triceps suralis.

References

Hypothesis

using SYBR green fluorescent stain (Applied Biosystems) in a sequence detection system (GeneAmp 5700, Applied Biosystems)\(^2\).

**Connective tissue density analysis**
The microscope slides were stained with Masson’s trichrome. We used a dot reading planimeter; and the counting was performed in squares of 2500 \(\mu\)m\(^2\) with 56 intersecting straight lines. Coinciding dots from the endomyosium and perimysium were counted in 5 fields/section of 5 sections/animal, a total of 1400 dots/animal. Thus, connective tissue density (relative area) was the result of the total coinciding dots on intersecting straight lines divided by the total number of dots\(^2\).

**Collagen birefringence analysis**
Microscope slides of the soleus muscle section of 10 \(\mu\)m were embedded in distilled water \((n_s = 1.333)\) for 30 minutes, and were covered with cover slips containing water at each interface.

Connective tissue birefringence was measured using Zeiss microscope with polarised light using a 10× objective lens with monochromatic light and 1/4 \(\lambda\) Sénarmont compensator. The optical delaying between the polarised lines that represents the macromolecular aggregation, and orientation state of the collagen fibres was also assessed. One hundred measurements at different areas of each muscle section were obtained by considering the embedding solution to provide collagen fibres heterogeneous distribution in each muscle. Collagen fibres were oriented at 45° for measurement\(^2\).

**Analysis of matrix metalloproteinases**
Muscle samples were treated as described by Cleutjens et al. The samples were homogenised and incubated in 0.5 ml of extraction buffer [cacodylic acid 10 mM with pH 5.0; NaCl 0.15 M; ZnCl\(_2\) 1 mM, CaCl\(_2\) 20 mM, NaN\(_3\) 1.5 mM; Triton X-100 0.01% (v/v)] at 4°C for 24 hours, and were centrifuged (10 minutes, 13,000 \(\times\) g at 4°C). The samples containing a pool of five separated areas of each muscle received 10 \(\mu\)g total proteins to increase their concentration, and resolved by SDS 10% and gelatin 1 mg/ml concentration gel electrophoresis. To remove the SDS after electrophoresis, the gels were washed in 2.5% Triton X-100 and incubated in a substrate buffer (Tris-HCl 50 mM pH 8.0; CaCl\(_2\) 5 mM, Na\(_2\)NO\(_3\) 0.02%) at 37°C for 20 hours. Gels were stained with Coomassie Blue Brilliant R–250 (Bio-Rad) for 90 minutes and destained in methanol:acetic acid:H\(_2\)O (4:1:5, v:v:v). Gelatinolytic activity was observed as light bands in the stained gel. These gels were photographed with Canon G6 Power Shot 7.1 mega pixels digital camera and the mean band intensity was analysed using the Gene Tools software. Data were expressed as MMP-2 active form and concentration (total integrated optical density for intermediate and active MMP-2 proenzyme).

**Statistical analysis**
Statistical analysis was performed using SPSS software v17.0, and the Shapiro–Wilks test was used for data normality. If the normality premises are confirmed, analysis of variance (ANOVA) and Duncan post hoc test were used to study the differences between groups. For all calculations, a 5% \((p < 0.05)\) significance level was established.

**Discussion**
Muscle atrophy and shortening are common problems in traumatology and orthopaedic rehabilitation. Despite the lack of evidence concerning its effects, diacutaneous fibrolysis has been used to treat such conditions. Therefore, further research is necessary to understand diacutaneous fibrolysis molecular and morphological mechanisms that affect muscle plasticity and ECM remodelling.


