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Remobilization through stretching improves gait recovery in the rat

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ABSTRACT

Remobilization of a previously immobilized rat right hindlimb in the ankle plantar-flexion-shortened position by free movement alone or associated with intermittent passive stretching was assessed by analysis of gait variables and dorsiflexion range of motion. The variables were related with the expression of extracellular matrix proteins and the addition of serial sarcomeres. Sixty-four female Wistar rats were divided into 8 groups: immobilized, free for 10 days, immobilized/stretched/free for 1, 3 or 10 days, immobilized/free for 1, 3 or 10 days. Gait variables, range of motion, serial sarcomeres number, localization and staining intensity of fibronectin, and expressions of types I and III collagen were analyzed. The hypokinesia changed the functional variables of gait, reduced the dorsiflexion range of motion (ROM), increased the number of fibers with intracellular fibronectin/total number of fibers (FIF/TNF), and decreased the expression of the type I collagen. After three days, morphological changes were exacerbated and the number of serial sarcomeres was increased in both groups, immobilized/stretched/free and immobilized/free. Functional impairment, ROM restriction and increased FIF/TNF were also observed. Despite the above described alterations, 10 days of stretching program increased the effectiveness of remobilization leading to recovery of the abnormalities observed in the muscle.

Introduction

Immobilization is frequently used to treat skeletal muscle injuries, but may induce alterations such as: muscular atrophy and reduction of muscle extensibility, strength, and resistance that tend to increase muscle-tendon fibrosis (Williams and Goldspink, 1984; Kannus et al., 1998; Mattiello-Sverzut et al., 2006) and also cause ligament changes and joint stiffness (Noyes, 1977; Loitz et al., 1989). These events are related to changes in the synthesis and degradation of muscle proteins and collagen produced by fibroblasts in the extracellular matrix (ECM) (Karpakka et al., 1990; Ahtikoski et al., 2001) with consequent connective tissue disorganization that directly affects the functional performance of the limb.

External stimuli, such as neuromuscular electrical stimulation (Carvalho et al., 2008), early mobilization (Hwang et al., 2006), eccentric training (Cornachione et al., 2011), or techniques such as stretching exercises (Polizello et al., 2009), may promote tissue recovery in the post-immobilization period. The passive muscle-stretching technique is one of the procedures for remobilization when the range of motion (ROM) is limited. The general goal of a stretching program is to reestablish the ROM of each joint to its normal pattern and to restore the mobility of the soft tissues that surround the joints so as to improve their functional performance (Weldon and Hill, 2003). Such results can be obtained regulating the area of the connective tissue and serial sarcomere loss (Coutinho et al., 2004). Stretching has also been reported to cause muscle hypertrophy (Coutinho et al., 2006; Cornachione et al., 2008; Secchi et al., 2008; Benedini-Elias et al., 2009). However, other studies have shown that the longitudinal passive tension of stretching seems to produce degenerative injuries in the muscle tissue when the stretching was applied during the process of immobilization (Mattiello-Sverzut et al., 2006; Gomes et al., 2007). Nevertheless, training of rats with previously immobilized limbs

Abbreviations: BSA, bovine serum albumin; C, free for 10 days/control; ECL, enhanced chemiluminescence; ECM, extracellular matrix; FIF/TNF, the number of fibers with intracellular fibronectin/total number of fibers; H.E., hematoxylin–eosin; I, immobilized; $IF_{(1)}$, immobilized and free for 1 day; $IF_{(3)}$, immobilized and free for 3 days; $IF_{(10)}$, immobilized and free for 10 days; $IS_{(1)}$, immobilized and stretched for 1 day; $IS_{(3)}$, immobilized and stretched for 3 days; $IS_{(10)}$, immobilized and stretched for 10 days; PBS, phosphate-buffered saline; PL, print length; ROM, range of motion; TBST, Tris-buffered saline with Tween 20; TS, total spread of toes; *vs.*, *versus*.

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with stretching exercises for 21 days produced few signs of tissue injury (unpublished observations, A.C. Mattiello-Sverzut).

The cytoskeletal network is a dynamic system constituted by filaments that connect the sarcolemmal proteins to ECM components as well as to intracellular organelles and the nucleus. This continuous network is involved in several functions, including: cell integrity, force transmission, mechanochemical signaling, and integration of organelle structure and function (Capetanaki et al., 2007). Restructuring processes related to filament protein expression after stretching stimulation are poorly understood. Differences in muscle stress properties related to collagen properties and muscle functions have been reported (Kovanen et al., 1984). Slow-twitch muscles, such as the soleus muscle, have relatively greater concentrations of collagen, which correlate with higher ultimate tensile strength, than do fast-twitch muscles such as the rectus femoris (Kovanen et al., 1984).

The enzymatic activities of prolyl-4-hydroxylase, which is involved in the synthesis of collagens, has been shown to increase during tissue repair, and the molecules thus formed provide adequate force distribution and support to the tissue (Ahtikoski et al., 2001; Kovanen, 2002). In parallel, in the initial phase of ECM restoration, the fibroblast adhesion support is provided by fibrin and fibronectin (Grinnell et al., 1980), both of which may be associated with the preexisting collagens (Kurkinen et al., 1980). However, the presence of intracellular deposits of fibronectin has been thought to indicate a loss of the cellular membrane integrity (Fridén et al., 1991).

The functional repercussions of intermittent manual stretching on the gait of previously limb immobilized animals also remain unclear. The integrity of the muscle length is essential for the correct performance of the different stages of the gait, and the fibers of the plantar flexor and dorsiflexor muscles are constantly recruited or stretched to allow their proper function (Varejão et al., 2002). However, the evolution over time of the relationship between the gait functional variables and the morphological variables, with reference to the dynamics of the muscle fiber length and the structural aspects of the ECM (which mechanically limit the joint ROM), has not yet been investigated in the context of rehabilitation after limb immobilization.

The objective of the present study was to examine the effectiveness of post-immobilization therapy including intermittent manual passive stretching in association with free movement during 10 days rehabilitation by analyzing the kinetic-functional and morphological variables of the hind limbs of female rats. We measured and recorded gait variables, the dorsiflexion range of motion of the ankle joint, and the addition of serial sarcomeres into the fibers of the soleus muscle of female rats previously immobilized in the ankle plantar-flexion-shortened position. These data were correlated with analyses of alterations in the morphology and the localization and staining intensity of ECM proteins of this muscle.

Materials and methods

Handling of animals and experimental procedures

This study was approved by the Ethics Committee for Animal Research of the Ribeirão Preto Medical School (No. 129/2009). Sixty-four female Wistar rats (weighing approx. 250 g) were divided into 8 groups: Immobilized (I), Free for 10 days/control (C), Immobilized and Stretched for 1 day ($IS_{(1)}$), Immobilized and Stretched for 3 days ($IS_{(3)}$), Immobilized and Stretched for 10 days ($IS_{(10)}$), Immobilized and Free for 1 day ($IF_{(1)}$), Immobilized and Free for 3 days ($IF_{(3)}$), and Immobilized and Free for 10 days ($IF_{(10)}$). The animals were kept four per plastic cage (41 cm × 34 cm × 16 cm) with free access to water and food pellets. The immobilization device was prepared with number 6 stainless steel mesh, cotton, impermeable surgical tape (Nexcare, 3M Health Care, St. Paul, MN, USA), micropore tape (3M Health Care), adhesive tape (3M Health Care), silver tape (3M Health Care), viscolycra fabric strips and a stapler. The upper part was similar to a T-shirt and made of viscolycra fabric. The lower part, divided into anterior and posterior sections, consisted of stainless steel mesh with the margins wrapped with impermeable surgical tape. The anterior section was also wrapped with cotton lining to protect the anterior surfaces of the immobilized limb and hip. Next, the upper and lower portions of device were joined with staples (Coutinho et al., 2002; Benedini-Elias et al., 2009). The rats of groups I, $IS_{(1)}$, $IS_{(3)}$, $IS_{(10)}$, $IF_{(1)}$, $IF_{(3)}$ and $IF_{(10)}$ were anesthetized intraperitoneally with sodium thiopental (Thiopentax, Cristália, Itapira, São Paulo, Brazil) and had their right hindlimb immobilized in full plantar flexion in order to maintain the soleus muscle in a shortened position. The animals could freely move their head and forelimbs.

After immobilization for 10 days, the animals from group I were sacrificed with an intraperitoneal injection of excess sodium thiopental (Thiopentax, Cristália, Itapira, São Paulo, Brazil); the animals from groups $IF_{(1)}$, $IF_{(3)}$, and $IF_{(10)}$ were allowed to move freely in their cages for 1, 3, or 10 days, respectively, and the animals from groups $IS_{(1)}$, $IS_{(3)}$, and $IS_{(10)}$ were subjected to a program of intermittent manual passive stretching with release in their cages between interventions for 1, 3, or 10 days, respectively. The manual passive stretching was performed by applying manual force to the plantar region in the dorsal flexion direction, stretching the soleus muscle of the right hindlimb. This stretching program comprised a daily series of 10 repetitions for 30 s each at 30-s intervals, measured with a chronometer (Mattiello-Sverzut et al., 2006; Polizello et al., 2009). Before the period of immobilization and during the remobilization programs (before and after the intervention), the dorsiflexion ranges of motion of the right ankle joints of the animals were measured using a finger goniometer (Carci, São Paulo, São Paulo, Brazil). The functional parameters of the gait of the rats were determined from footage of the animals on a treadmill (Insight®, Ribeirão Preto, São Paulo, Brazil). The images thus generated allowed the capture of the footprints at the exact moment when the hindlimbs were in the double-support phase, in which the right hind limb is in the phase of weight acceptance and the left in toe-off. These images were analyzed using the analysis software modified by Yamasita et al. (2008). The parameters measured were the print length (PL) and the total spread of the toes (from the 1st to the 5th; TS), both suggested by Bain et al. (1989). Pre-immobilization data on the dorsiflexion range of motion and the functional gait parameters were used to generate reference values for each animal. For this reason, we did not obtain data from the animals of groups I and C.

At the end of the experimental procedures for each group, the rats were weighed and then sacrificed by an overdose of sodium thiopental anesthetic. Subsequently, the right hindlimb was positioned in hip external rotation, knee extension, and tibiotarsal joint neutral position for measurement of the length between the proximal and distal muscle-tendon junctions using a digital caliper (Marberg, São Paulo, Brazil). Later, the soleus muscle was dissected and placed on cork coated with Parafilm (Pechiney Plastic Packaging Company, GuangZhou, China), and the tendon extremities were fixed with pins (the original length of the muscle was maintained using the digital caliper). Following this process, the medial portion was divided into two parts: one part was immersed in talcum powder and frozen in liquid nitrogen for histochemical and immunohistochemical analysis. The other part was washed in 0.9% sterile saline solution, dried on filter paper, frozen in liquid nitrogen, and stored at −80 °C until the time of protein extraction by Western blot. The lateral portion was kept in the cork-pin