Life Sciences 137 (2015) 158–163

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Sensorial, structural and functional response of rats subjected to hind limb immobilization

Francisco Fleury Uchôa Santos-Júnior, Alana de Freitas Pires, Natália Matos Ribeiro, Vanessa Azevedo Mendonça, Juliana Osório Alves, Paula Matias Soares, Vânia Marilande Ceccatto *, Ana Maria Sampaio Assreuy

Instituto Superior de Ciências Biomédicas, Universidade Estadual do Ceará (UECE), Fortaleza, CE, Brazil

ARTICLE INFO

Article history:
Received 16 October 2014
Received in revised form 16 June 2015
Accepted 21 July 2015
Available online 29 July 2015

Keywords:
Hypokinesia
Nociception
Hot plate
Von Frey
Skeletal muscle

ABSTRACT

Aims: This study analyzed the sensorial, structural and functional response of rats subjected to paw immobilization.
Main methods: Animal pelvis, hip, knee and ankle were immobilized using waterproof tape during two weeks for assessment of sensorial response to thermal (hot plate test) and mechanical stimuli (Von Frey test), motor system structure (histology and radiography) and muscle function (soleus contractility).
Key findings: Disuse animals became more responsive to thermal stimuli (49%), although less responsive to mechanical challenge (58%). Disuse animals showed local injuries such as reduction in muscle fiber diameter (16.7% in gastrocnemius, 5.7% in soleus), contractile activity (55% of the control maximal tonic contraction) and tibia cortical thickness (9.3%), besides increased nitrite:protein ratio, suggestive of protein degradation. Disuse also evoked systemic adaptations that include increase in serum lactate dehydrogenase (36.1%) and alkaline phosphatase (400%), but reduction in calcium (8.4%) and total serum protein (5.5%), especially albumin (34.2%).
Significance: Two weeks of functional paw disuse leads to local and systemic harmful adaptive changes in sensorial and structural systems. This study brings new insights into nervous and motor system mechanism associated with therapeutic limb immobilization in muscle and skeletal pathological conditions.

1. Introduction

Immobilization is a common clinical practice, especially in the treatment of fractures and pain conditions. However, even short periods of immobilization carry multiple risks, particularly muscle atrophy [4]. This procedure also has other disadvantages, including bone loss, swelling, joint stiffness, innervation and circulation disorders [1], connective tissue accumulation, and histological [6] and functional alterations in muscle fibers [30].

The contractile activity of isolated skeletal muscles is diminished during the immobilization process [30], accompanied by reduction in amino acid transporters [11]. Accordingly, rat gastrocnemius atrophy induced by denervation or spinal cord isolation stimulates atrogene expression similar to that observed in systemic conditions such as untreated diabetes, tumor implantation and renal failure. These changes may influence adaptations to the loss of muscle contractile activity [23]. In addition, muscles kept in disuse are more sensitive to hormonal catabolic signals, directing metabolic pathways toward mass and protein loss [2].

Bone loss is another complication of disuse, occurring either locally or systemically [21]. It is characterized by increased bone resorption by osteoclasts and reduced bone mass formation by osteoblasts [25]. Long-term disuse occurs in three stages; rapid bone loss begins immediately after immobilization, followed by slower but prolonged loss initiated around the 12th week, and finally, a stabilization period that results in 40–70% reduction of original mass [10].

Two experimental models, suspension and immobilization, are currently used to induce muscle atrophy and simulate bed restriction and low functional activity [13]. A comparative study showed that both models produce significant reduction in soleus muscle mass, isometric contraction duration, and peak tetanic tension [24]. Nervous system alterations have been demonstrated in a two-week immobilization model [1]. However, thermal and mechanical muscle hyperalgesia may also occur following disuse. This response is still poorly studied, as most of the investigations have been evaluated for more than three weeks of disuse [30]. Thus, we hypothesized that these changes are also present after two weeks of paw immobilization. For better understanding of the disuse pathophysiology, the present study aimed to analyze the sensorial response to thermal and mechanical stimuli as well the motor

* Corresponding author at: Laboratory of Biochemistry and Genic Expression — Superior Institute of Biomedical Sciences, Av. Dr. Silas Munguba, 1700 Campus do Itaperi, Fortaleza, CE 60714-903, Brazil.
E-mail addresses: vania.ceccatto@uece.br, http://www.uece.br/cmacf (V.M. Ceccatto).

http://dx.doi.org/10.1016/j.lfs.2015.07.020
0024-3205/

system structure and function in rats subjected to paw immobilization for two weeks.

2. Materials and methods

2.1. Animals

Eight-week-old female rats (*Rattus norvegicus*, Wistar) with an average body mass of 200–230 g were given ad libitum access to food and water. This study was approved by the Ethics Committee for Animal Use of UECE (CEUA no. 10725887-0).

2.2. Immobilization model

Rats were anesthetized with 60 mg/kg ketamine + 8 mg/kg xylazine before immobilization. Waterproof tape (10 cm wide; Cremer™, Santa Catarina, Brazil) was utilized to immobilize the pelvis. Hips and knees were immobilized in extension, and ankles in plantar flexion. Cotton strips (4 cm wide) were placed at the joints to prevent the formation of pressure ulcers. The right hind limbs were firmly bandaged with 2×15 cm^2 tape strips and maintained for two weeks, a procedure that does not interfere with the movement of rats within the cage or the water and food access. Animals were weighed before and after two weeks of immobilization [24].

2.3. Evaluation of thermal and mechanical responses

The sensorial response to thermal stimuli was evaluated in the hot plate test. For this, animals were placed at 55 ± 0.5 °C for up to 25 s [5]. The reaction time to thermal stimulus (time to jumping, or licking or shaking of hind paws) was registered 3 h of tape removal [12].

The sensorial response to mechanical stimuli was evaluated in the Von Frey test to investigate allodynia, a response to a non-nociceptive stimulus, or hyperalgesia, an increase in pain sensitivity. For this, animals were individually placed in clear acrylic boxes with raised platforms of wire mesh to allow access to the ventral surface of hind paws. The frequency of paw withdrawal in response to ten applications of flexible Von Frey filaments (constant pressure of 0.8 g) was measured [32]. An additional control group was assessed in which eight animals received subcutaneous injection of the inflammatory agent λ-carrageenan (Sigma Chemical Co. — St. Louis, MO, USA) dissolved in sterile saline (300 μg/100 μL) in order to induce paw edema [9]. Three hours later the mechanical response was evaluated in the Von Frey test and the paw volume (mL) by plethysmometry.

2.4. Tissue analysis

Blood was collected by retro-orbital puncture for hemogram and biochemical analysis of alkaline phosphatase, lactate dehydrogenase (LDH), creatinine, glucose, total protein, albumin, calcium, and lactate serum levels using LABTEST kits (Labtest Diagnóstica, Lagoa Santa — MG, Brazil).

After euthanasia by CO_2 inhalation, the tibia was excised for examination by X-ray (45 kV, 4 mA s) and microscopy. Each bone was evaluated at 3 locations: proximal epiphysis, diaphysis and distal epiphysis. The equipment tube was vertically positioned, and anatomical samples were placed 80 cm from the emission focus with the anterior surface facing upwards under the exposure time of 0.04 s (35 × 43-cm film, Kodak™). The film was digitized using photographic camera (Olympus 1040; Olympus™) with 5 megapixel resolution. Optical densities (O.D.) were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Tibia thickness was measured using longitudinal sections of the middle third (diaphysis).

For histological analysis, performed by a single examiner, soleus and gastrocnemius were weighed, fixed in 10% formol for 24 h, subjected to dehydration in crescent alcoholic series, diaphanized in xylol, impregnated in paraffin and melted at 60 °C to form blocks. Fiber diameter was measured (3 slides/muscle — 5 μm thick) and, in each section, 2 areas (50 fibers/1000 μm radius) were randomly selected and stained in cross-section with hematoxylin & eosin and analyzed by optical microscopy (Zeiss Primo Star/Pixel link PLA 662, 4 × 0.10/ImageJ). Muscles were further macerated to determine protein [3] and nitrite [22].

2.5. Evaluation of soleus contractile response

Pieces of soleus (3–5 mm) were mounted for tension recording (1 g) in a 10 mL organ bath filled with modified Krebs solution (113 NaCl; 2.19 $CaCl_2$; 25.0 $NaHCO_3$; 0.44 $MgSO_4$; 1.18 KH_2PO_4; 0.03 EDTA; 11.0 glucose mM). After equilibrium of 45 min (37 °C, 95% O_2, 5% CO_2; pH 7.4), tissues were challenged with caffeine (50 mM), and the contractile response (isometric tension, in g) was measured for 30 min (PowerLab ADInstruments) and analyzed by computerized data acquisition system (Chart 4.2) [30]. Data were expressed as percentage of maximal contraction or as area under the curve (arbitrary units).

2.6. Statistical analysis

The data were expressed as mean ± S.E.M. ($n = 6–16$). Student's t-test was used for independent samples; one-way ANOVA (Dunnett's post-test) and two-way ANOVA (Bonferroni's post-test) for protocols of muscle contractility; Spearman correlation was applied to compare histology, biochemical parameters and muscle mass. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of disuse on the rat thermal and mechanical response

Two weeks of paw immobilization diminished animal responses to mechanical stimulus. The frequency of paw withdrawal was reduced by 58% in the disuse group (3.13 ± 1.5%) compared to the control group (7.5 ± 1.7%) (Fig. 1A). Both the disuse and control groups differed from the carrageenan group (72.9 ± 7%; $p < 0.0001$), used as positive inflammatory and hyperalgesic control, exhibited hyperalgesic response to a non-noxious stimuli (Von Frey 0.8 g filament). Moreover, the immobilization process failed to induce inflammation since the paw volume of the disuse animals (1.22 ± 0.04 mL; $n = 16$) was not different from that of the control animals (1.26 ± 0.03 mL; $n = 16$; $p = 0.343$). Disuse and controls differed from the edematous paws induced by carrageenan (3.84 ± 0.14 mL; $p < 0.0001$). In the hot plate test, the animals' reaction time to thermal stimulus was decreased by 49% in the disuse group (3.39 ± 0.32 s) compared to controls (6.62 ± 0.7 s) (Fig. 1B).

3.2. Hematological and structural alterations derived from disuse condition

The immobilization procedure led to increase in the levels of serum alkaline phosphatase (400%) and LDH (36.1%), but reduction in calcium (8.4%), total protein (5.5%) and albumin (34.2%) relative to control animals. However, serum lactate, glucose, creatinine and other hematological parameters, including hematocrit, hemoglobin and blood cells (platelets, leucocytes and erythrocytes) were unaltered. Notably, the low calcium content in the disuse group was still within the normal range (Table 1).

Body mass in the disuse group was reduced by 13.8% after two weeks of immobilization compared to controls. The wet weight of gastrocnemius and soleus muscles were also diminished by 38.1% and 35.7%, respectively. In addition, the nitrite:protein ratio was augmented in the soleus (46.6%) and gastrocnemius (83.7%) as a result of paw immobilization (Table 2).

The histological profile of control gastrocnemius showed a distribution frequency of 70–90 μm in 66% of the analyzed fibers. However, in