EXERCISE AND SPORTS SCIENCES

SWIMMING INFLUENCE ON THE EXPRESSION OF MYOGENIC REGULATORY FACTORS DURING SKELETAL MUSCLE REPAIR OF RATS

ORIGINAL ARTICLE

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ABSTRACT

Background: Skeletal muscle has the ability to adapt to several stimuli, such as contractile activity as well as direct and indirect damage. Aquatic therapy has been used in the rehabilitation of musculoskeletal disorders. In addition, it has demonstrated positive results in the therapeutic process and preventing several diseases.. Objective: The aim of the present study was to analyze the effect of swimming on the expression of the myogenic regulatory factors MyoD and myogenin during the skeletal muscle repair process in rats following cryoinjury. Methods: Forty Wistar rats were randomly divided into four groups: 1) Control; 2) Sham – non-muscle damaged, submitted to procedure for exposure of the tibialis anterior (TA) muscle; 3) Cryoinjured; and 4) Cryoinjured and submitted to swimming. Analyses were carried out at 7, 14 and 21 days. Cryoinjury was performed with two applications of the flat end of a metal rod previously cooled in liquid nitrogen directly to the belly of the TA muscle. The protocol consisted of 90-minute swimming sessions six times a week. At the end of the protocol, the animals were euthanized and the TA muscles were removed. Total RNA was extracted using the TRIzol reagent. Next, cDNA was obtained to perform real-time PCR using specific primers for MyoD and myogenin. Results: The results showed a reduction in the expression of myogenin in the groups cryoinjury with $p \leq 0.01$and without $p \leq 0.01$ swimming after 7 days, and in group cryoinjury with swimming ($p \leq 0.05$) after 14 days respect to the control groups and "sham", respectively. There were no differences between groups cryoinjury with ($p > 0.05$) and without ($p > 0.05$) swimming. Regarding the expression of MyoD there was no difference between the groups. Conclusion: Swimming did not affect the expression of myogenic regulatory factors during the skeletal muscle repair process in rats following cryoinjury.

Keywords: swimming, repair, skeletal muscle, MyoD, myogenin.

INTRODUCTION

Skeletal muscles are dynamic muscles which have an extraordinary ability of adaptation when exposed to varied stimuli such as contractile activity, direct damage (laceration, contusion and sprain) and indirect damage (ischemia and neurological dysfunction), to more suitably meet the functional demand[1-4]. This ability is named plasticity[5-6]. In normal circumstances, the adult skeletal muscle of mammals is a stable tissue with low turnover, that is, low remodeling without dramatic alterations in their phenotype characteristics[6].

After an injury, the muscle has the ability to initiate a highly organized repair process in order to prevent loss of muscular mass and restore its normal functions. This process is similar to the myogenesis, namely formation of muscular tissue; however, the cells which initially participate are satellite cells rather than myogenic pregenitors[1,2,7,8]. After the activation, the satellite cells suffer a set of stages which involve proliferation, differentiation and fusion of myoblasts in the muscular fibers in order to repair muscular damage or constitute a new fiber[1,9]. In the different myogenesis stages, the cells express distinct myogenic regulatory factors factors involved in this process such as MyoD and myogenin. MyoD is directly related to activation, proliferation and differentiation of satellite cells, while expression of myogenin is related to the terminal differentiation[2,10-14].

Some studies have recently demonstrated a direct relation between expression of myogenic regulatory factors and performance in physical exercises in the presence or not of muscular injury[15,16].

Flynn et al., 2010[15], demonstrated that knock-out mice for the myogenin gene presented performance significantly better than the controls when submitted to high and low intensity exercises on treadmill, suggestion hence that myogenin plays a critical role as a high-level transcriptional regulator to control the energy balance between aerobic and anaerobic metabolism in adult skeletal muscle. Meadows et al., 2011[16] demonstrated that mdx knock-out mice for the myogenin gene presented higher resistance to exhaustive exercise which was associated with increase of neuron synthase nitric oxide expression (nNOS), related to muscular atrophy and fatigue ad suggest that reduction in myogenin expression in other muscular diseases could partially restore muscular function.

Currently, there is growing concern in providing faster and better-quality muscular repair process (regeneration), especially at conditions as repair after injuries in athletes, transplant, atrophy by disuse, remaining in space, among others[4]. Many injury kinds and models have been proposed to examine the regeneration process of the skeletal muscle, including damage by contusion, icing and use of chemical substances and by poisoning[17-20]. The cryoinjury model is able to induce injury and subsequent regeneration in a

delimited area of the muscular belly, besides causing a clean and easily reproducible injury[21], which mimes muscular injuries common in sports practice Aerobic exercise is widely used in rehabilitation of musculoskeletal dysfunctions and in that context swimming has gained increasing attention; however, the data in the literature concerning its effects and protocols to be used after the different kinds of injury are not well-established.

Physical activity in aquatic environment presents many physiological effects which may bring benefits to the tissue repair process, with emphasis on increase of peripheral circulation and consequent increase in oxygen and nutrients supply to the active muscle, increase in blood and lymphatic return, reduction of edema by the activity of hydrostatic pressure and reduction of sensitivity of the nervous terminals, which added to all the others, cause muscular relaxing[22].

Thus, the aim of the present study was to assess the swimming effect on the expression of the myogenic regulatory factors MyoD and myogenin during repair of the skeletal muscle of rats after cryoinjury.

MATERIALS AND METHODS

The methodology used in this study was designed according to the resolutions 196/96 of the National Health Board and approved by the Ethics in Research Committee of UNINOVE (number AN 0013/2009).

Animals

40 male Wistar rats, mean age of 12 ±1.2 weeks and weight range between 200±15g, kept at the animal facility of the Nove de Julho University (UNINOVE) were used in this experiment. The animals were kept in proper plastic boxes, room temperature of (22°C), relative humidity (40%), controlled luminosity with 12-h cycle (light/dark) and food and water ad libitum.

The animals were randomly divided in 04 experimental groups: Group (1) – Control (n=5); Group (2) – Sham: (the animals were only submitted to the exposure of the tibialis anterior muscle procedure (TA)) (n=5); Group (3) – Cryoinjured, euthanized after 7 (n=5), 14 (n=5) and 21 days (n=5); Group (4) – Cryoinjured and submitted to swimming, euthanized after 7 (n=5), 14 (n=5) and 21 days (n=5).

Cryoinjury procedure

The animals were anesthetized with intraperitoneal injection of the general anesthetic with base of ketamine (Dopalen) and xylazine (Anasedan). Serynges brand name BD 100 Units with needle BD Ultra-Fine®, insulin model with needle Ultra-Fine® (regular), length: 12.7 mm, caliper: 0.33 mm and bisel trifaceted were used to apply the anesthetic. Each animal was anesthetized using a ketamine 10% (0.2 ml/100 grams of the animal) and xylazine 2% (0.1 ml/100 grams of the animal) mixture. The cryoinjury model was used according to description by Miyabara et al[21]. The TA muscle was surgically exposed and the cryoinjury was performed with two applications (during 10 seconds each), using a metal rod of flat extremity previously cooled in liquid nitrogen, directly on the ventral surface of the muscle. The metal rod used in the TA muscle was 0.4 x 0.4cm and 0.4 x 1cm. After the procedure, the suture of the incised areas was performed using polyamide thread (6.0) and the animals were kept in cages with heating to avoid hypothermia. At the end of the swimming protocol, the animals were euthanized by the anesthetic overdose and the TA muscles were removed.

Swimming protocol

Previously to the beginning o the swimming protocol, the animals were adapted for a period of six days, following the protocol proposed by Takeda et al[23]. On the day following the end of the adaptation, the cryoinury procedure was performed and the animals were kept at rest for 3 days so that skin healing could take place and the swimming protocol could be initiated without risk of infection or complications as scar dehiscence.

The animals were submitted to the swimming protocol in PVC compartments with 24 cm of diameter and with water at 50 cm deep and mean temperature of 33±1 ºC. The animals swam for 90min/day, 6 weekly times[23] totalizing 03, 09 or 15 sessions for the groups 7, 14 and 21 days respectively, since a 3-day period was waited so that the animal's skin could heal. During the experimental protocol the water was moved and the animals had weight attached to their tails (up to 20% of their body weight) to avoid floating and guarantee hence the performance of the proposed training[24]. At the end of the proposed training, the animals were euthanized by anesthetic overdose and the TA muscles were removed for extraction of total RNA and analysis of the gene expression by real-time PCR.

Extraction of total RNA

80-100mg of the TA muscle removed was used for this procedure. The tissue was homogenized in TRIzol reagent (Invitrogen, Brazil) and the total RNA isolation was obtained following the manufacturer's recommendations. In order to verify the success of the extraction procedure, the total RNA samples were analyzed by formaldehyde 1% gel electrophoresis.

Total RNA was quantified by spectrometry in 260nm and all samples were treated with DNAse (Invitrogen, Brazil) to avoid contamination by genomic DNA. All solutions used for the described procedures were prepared with free water of RNAse treated with 0.01% of DEPC (Dimetil pirocarbonate) and the plastic and glass material also received treatment with RNAse.

cDNA and quantitative PCR (polymerase chain reaction) synthesis

cDNA synthesis and real-time PCR were used for analysis of the gene expression. Reverse transcription (RT) was performed in a 200 µl reaction, in the presence of 50 mM Tris-HCl, pH 8.3, 3mM MgCl2, 10 dithiothreitol mM, 0,5 mM dNTPs and 50ng of random primers with 200 units of Moloney murine reverse transcriptase of the leukemia virus (Invitrogen, Brazil). The reactions were kept at 20° C conditions for 10 min, 42° C for 45 min and 95° C for 5 minutes. One microliter of the RT reaction was used for the real-time PCR performance.

The real-time PCR was performed with the SYBRGreen kit (Applied Biosystems, USA) in 7000 Sequence Detection System (ABI Prism, by Applied Biosystems, Foster City, CA). The conditions used were 50°C for 2min, 95°C for 10 min., followed by 40 cycles

at 95°C for 15 seconds and 60°C for 1 min. The experiments were performed in three copies for each data point. Abundance of MyoD and myogenin RNAm was quantified with a relative value in comparison with the expression of the constitutive gene (GAPDH).

The primers used for the real-time PCR reaction were: GAPDH (GenBankTM access number NM 017008) sense 5'- TGCACCACCAACTGCTTAGC -3' and anti-sense GCCCCACGGCCATCA -3'; MyoD[25] - sense 5' GGA GAC ATC CTC AAG CGA TGC and anti-sense AGC ACC TGG TAA ATC GGA TTG (amplification product: 80pb); Myogenin[26,27] – sense 5'-ACTACCCACCGTCCATTCAC-3' and anti-sense 3'-TCGGGGCACTCACTGTCTCT-5' (amplification product: 233pb).

The quantitative values for RNAm of MyoD, myogenin and GAPDH were obtained from the number of cycles (CT – threshold cycle), in which there is increase of the signal associated with exponential increase of the PCR products. The fusion curves were generated at the end of each run to guarantee uniformity of the product. The level of relative expression of the target gene was normalized based on the GAPDH expression as endogenous control. ΔCt values of the samples were determined subtracting the mean value of Ct MyoD/myogenin RNAm Ct of the mean value of the GAPDH of inner control. Since it is rare to use ΔCt as relative data due to this logarithmic characteristic, the 2 ΔCt parameter was used to express data of relative expression.

STATISTICAL ANALYSIS

The RNAm MyoD and myogenin data were presented as mean ± standard deviation (SD) values. One-way ANOVA was used for comparisons between groups. Tukey test was used for determination of significant differences between the experimental groups. p ≤0.05 value was considered statistically significant. Data analysis was performed with help of the GraphPad Prism 4.0 statistical softwarer (GraphPad Software, San Diego, CA, USA).

RESULTS

The results evidenced that there was not significant difference in the RNAm MyoD expression between control (0.87±0.19; p>0.05) and "sham" (0.59±0.22; p>0.05) groups (figure 1). After 7 days, there was increase in the RNAm MyoD expression. In the cryoinjured group submitted to swimming (0.81±0.44; p>0.05) in comparison with the one which did not receive this treatment (0.49±0.14; p>0.05), but not significant. Finally, no significant difference was observed between the cryoinjured (0.53±0.28; p>0.05) and without swimming (0.39±0.21; p>0.05) groups assessed after 14 days and between the cryoinjured (0.41±0.11; p>0.05) and without swimming group (0.46±0.36; p>0.05) after 21 days (figure 1).

Concerning the myogenin expression, significant reduction in RNAm of this regulatory factor was found after 7 days in the cryoinjured groups with (1.00±0.23; p<0.01) and without swimming (1.02±0.26; p<0.01) and after 14 days in the cryoinjured with swimming group (1.09±0.26; p<0.05) when compared with the control (1.78±0.06) and "sham" (1.77±0.18) groups. In the cryoinjured without swimming (1.32±0.48; p>0.05) after 14 days and cryoinjured with swimming (1.62±0.31; p>0.05) and without swimming (1.30±0.10; p>0.05) after 21 days groups, the myogenin expression was similar to the control and "sham" groups (figure 2).

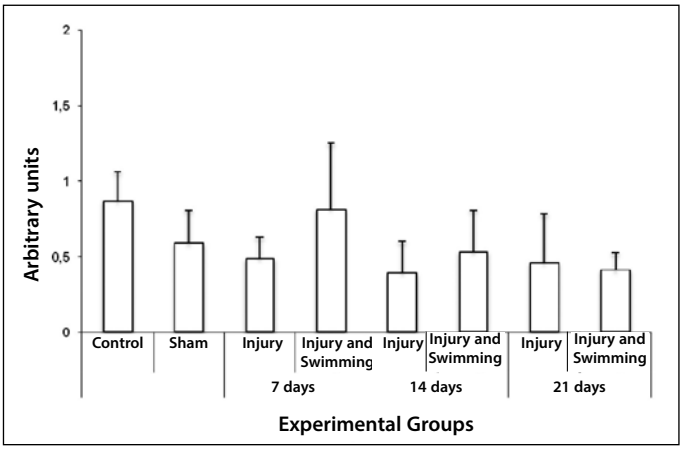

Figure 1. Comparative analysis of RNAm of MyoD in the experimental groups assessed (p ≤ 0.05).

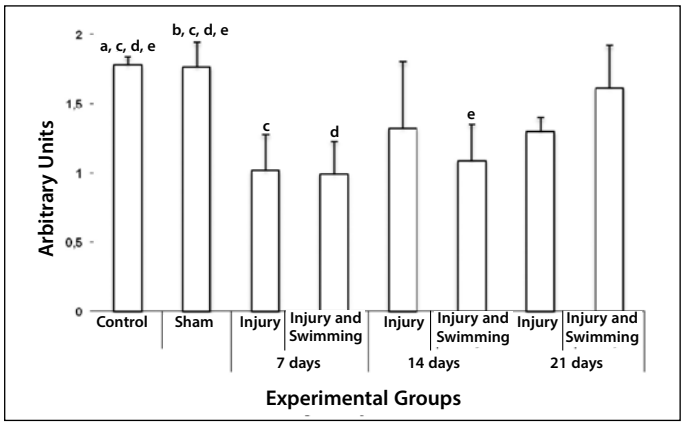

Figure 2. Comparative analysis of RNAm of myogenin in the experimental groups assessed. Same letters denote significant statistical difference (p ≤ 0.05).

DISCUSSION

Different therapeutic modalities are applied to provide a better quality and shorter muscular repair process[8,9]. Aerobic exercise is extensively used in rehabilitation of musculoskeletal dysfunctions. Therefore, we analyzed the effect of aerobic exercise (swimming) on the MyoD and myogenin expression during repair of TA muscles in rats. The results demonstrated that the aerobic exercise protocol used in the present study was not able to alter the MyoD and myogenin expression during the repair process in the analyzed periods.

MyoD is expressed in the initial phases of the muscle repair process and is involved in activation, proliferation and differentiation of satellite cells, while myogenin is late expressed and is related to differentiation of the myoblasts terminal[2,10-14,28]. Some studies have shown that the expression of these myogenic regulatory factors (MyoD and myogenin) also contributes to the hypertrophic growth of the skeletal muscle[29,30]. The MyoD expression is stimulated immediately after the muscular injury induced by bupivacaine, according to Marsh et al[31] the stimulation peak of this marker in muscles of rats occurs around at 5 to 10 days, and these authors evidenced complete regeneration after 21 days. Since the evaluation in the present study was performed at the 7, 14 and 21 days periods, it is possible that the expression peak of this marker occurred at any period previous or intermediate to the ones evaluated and had not been detected, but simply not significant increase in the expression of MyoD after 7 days of injury had been verified in the

group submitted to swimming compared to the group which did not perform aerobic exercise. However, these same periods of evaluation had been used in other studies with use of interventions different from in the present study [30,32].

Our results have also shown significant reduction in the expression of myogenin after 7 days, being this reduction equivalent to 44.11% in the injured group submitted to swimming and 42.8% in the group only injured. Moreover, reduction of 38.69% was observed in the injured group submitted to swimming after 14 days compared with the control group. Currently, some authors have demonstrated another clinical role of myogenin as a high transcriptional level regulator for control of the energetic balance between aerobic and aerobic metabolism in adult skeletal muscle and its suppression would increase resistance in physical exercise performance [15-16]. Thus, the reduction found in the expression of this myogenic regulatory factor in the groups submitted to swimming could be related to increase in that performance capacity.

It is worth mentioning that in a previous study performed by our research group, it was observed that swimming did not alter the morphological aspects of the TA muscle of rats under repair process after cryoinjury, being analyzed at 7, 14 and 21 days[32].

Mesquita et al[33] using aquatic training showed that it was not able to promote significant alteration in the expression of myosin heavy-chain isoforms. However, Sugiura et al[34] observed that animals submitted to aquatic training after a long immobilization period presented reduction of type 2B fibers and increase of 2D fibers in the muscles of rats, evidencing hence that aquatic therapy is an stimulus able to generate muscular remodeling for better adaptation when experiencing functional demands. Volpi et al[35] used aquatic exercise to verify its effect in the remodeling of the soleus muscle of rats after atrophy models by immobilization and evidenced that aquatic exercise was not able to totally revert the atrophy process caused by the immobilization. In the present study, we demonstrated that in a muscular repair situation, swimming was not able to influence the MyoD and myogenin expression in the analyzed periods.

The study of the expression of myogenic markers, such as MyoD and myogenin, involved in the activation and differentiation process of satellite cells in different therapies used in clinical practice for rehabilitation of patients after muscular injury, will be able to make us understand how these instruments will interfere in the muscular regeneration process. Thus, it becomes clear the need of further studies which bring greater contributions in this aspect, enabling the establishment of more efficient protocols to be adopted in the clinical practice.

CONCLUSION

It was concluded that swimming did not induce alterations in MyoD and myogenin expression. During the skeletal muscle repair process of rats after cryoinjury, although significant reduction in the myogenin expression has been observed in the groups submitted to swimming after 7 and 14 days compared with the control group.

All authors have declared there is not any potential conflict of interests concerning this article.

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