Treatment of Muscle Injury with Stem Cells – Experimental Study in Rabbits

Tratamento da lesão muscular com células-tronco – Estudo experimental em coelhos

Alex de Lima Santos1, Camila Gonzaga da Silva2, Leticia Siqueira de Sá Barreto2, Marcel Jun Sugawara Tamaoki1, Bruno Fiorelini Pereira3, Fernando Gonçalves de Almeida2, Flavio Faloppa1

1 Department of Orthopedics and Traumatology, Escola Paulista de Medicina, UNIFESP, São Paulo, SP, Brazil
2 Department of Surgery, Escola Paulista de Medicina, UNIFESP, São Paulo, SP, Brazil
3 Department of Biological Sciences, Campus Diadema, UNIFESP, São Paulo, Brazil

Address for correspondence Alex de Lima Santos, PhD in Medicine, Department of Orthopedics and Traumatology, Escola Paulista de Medicina, UNIFESP, São Paulo, Brazil (e-mail: alexdels@gmail.com).

Keywords
► mesenchymal stem cells
► regenerative medicine
► muscular diseases
► muscles
► regeneration

Abstract

Objective Histological and macroscopic evaluation of the healing process of acute lesions of the femoral rectus muscle using stem cells derived from adipose tissue-derived stem cells (ADSCs).

Method An experimental study was conducted with 18 hind legs of New Zealand rabbits, which were divided into three study groups according to the intervention to be performed. In group I, no surgical procedure was performed; in group II—SHAN, the experimental lesion was performed without any additional intervention protocol; in group III—Intervention, the addition of ADSCs was performed in the same topography of the experimental lesion. After the proposed period, 2 weeks, the material was collected and submitted to macroscopic and histological evaluation.

Results The quantitative analysis showed that the addition of ADSCs is related to the reduction of inflammatory cells in the 2-week evaluation (164.2 cells in group II – SHAN to 89.62 cells in group III – ADSC). The qualitative analysis of the slides with Picrosirius red, noticed an increase in orange/yellow fibers in group III – ADSC, which evidences a final healing process. The macroscopic evaluation found no difference between the groups.

Conclusion The use of ADSCs in the treatment of acute muscle injury presented histological advantages when compared to their non-use.
Introduction

Muscle injury represents approximately one third of injuries related to sports activity; it mainly affects the lower limbs and has an important relationship with withdrawal from sports activities.1–3 For an adequate diagnosis, we opted for a clinical evaluation, with the use of imaging tests reserved for diagnostic confirmation, qualification, and quantification of the lesion.4

There are some etiological factors with a well-established association with an increased risk of muscle injuries. Among them, we can mention age, previous muscle injury, ethnicity, overload, and imbalance of muscle forces.5 The therapeutic management of these lesions has not presented substantial changes over the last few years, and the RICE (rest, ice, compression, and elevation) protocol is the most widely used treatment.4,6,7

Even after performing an appropriate treatment protocol, the high rate of re-injury and prolonged absence from sports activities3,8 motivates the constant search for new therapies that can improve the results. Seeking to fill this space, the use of orthobiologics has been gaining space in the treatment of various orthopedic lesions, including muscle injuries.9

Among the available orthobiologics, the use of adult mesenchymal stem cells, especially those derived from adipose tissue, already presents consistent results in terms of differentiation capacity,10,11 rapid growth,12 ease of obtaining,13 good experimental results,14,15 and promising clinical results.16,17

Thus, in the search for alternatives for muscle repair, the present study proposes to evaluate the hypothesis that muscle healing can be optimized using adipose tissue-derived stem cells (ADSCs), in an experimental model of muscle injury reproduced in rabbits. It precisely aims at the histo-

logical and macroscopic evaluation of the healing process of acute lesions of the femoral rectus muscle using ADSCs.

Material and Method

Experimental Design

An experimental study was conducted with 9 pure New Zealand male rabbits, aged 28 to 32 weeks and with approximate weight between 3 and 3.5 kg. The animals were acquired from a commercial establishment and kept in the development center of experimental models for biology and medicine throughout the study. During this period, the animal was kept in an individualized environment, 12/12 hrs dark-light cycle, with food and water ad libitum. The hind legs of the animals (18 legs) were randomly divided (using specific software and opaque envelopes) in the study groups according to the intervention to be performed (Figure 1).

In the group I – control, the hind legs were kept intact, in group II–SHAN, the experimental lesion was performed without association with additional treatments, and in group III – ADSCs, the experimental lesion was performed and the addition of ADSCs to the lesion site, as a treatment intervention (Figure 1). The study had its initial version and subsequent reports approved by the ethics committee for the use of animals (CEUA, in the Portuguese acronym) of our institution and followed the guidelines for the use and management of animals proposed by our institution besides meeting the criteria proposed in the animal research: reporting of in vivo experiments (ARRIVE) guidelines.18

Procedures

To perform the experiments, whether fat collection, lesion protocol, or material collection, the animals were submitted...
to the following analgesic and anesthetic protocol: To start the procedures, the animal was submitted to analgesia and preoperative antibiotic therapy with tramadol (5 mg/kg) and terramycin (50 mg/kg); after 30 minutes anesthesia, ketamine (50 mg/kg) and xylazine (10 mg/kg) were started. As a method of postoperative analgesia, meloxican (0.5 mg/kg) and tramadol (5 mg/kg) were maintained until the end of the 3rd postoperative day, and these same medications were administered in case of pain or discomfort of the animal after this period. Evaluations regarding stress, discomfort, and pain were performed daily at the development center of experimental models for biology and medicine.

Experimental Model of Acute Muscle Injury
After an anesthetic protocol already presented, the animals with paws belonging to the group II—SHAN or to the group III—ADSC were submitted to trichotomy, antisepsis, asepsis, anterior skin incision in the thigh, diluvision by planes, and exposure of the femoral rectus throughout its extension (Figure 2A). Next, partial injury in the 1/3 middle of the femoral rectus was performed (Figure 2B), with coldblade, and marking of the extremities (Figure 2C) with nylon 6-0 (Nylon 6-0, Shalon, Alto da Boa Vista, GO, Brazil), at approximately 0.5 cm proximal and distal to the lesion. After performing the procedures and anesthetic recovery, the animal was encouraged to apply load to the limb, without restrictions.

ADSCs—Fat Collection to implant ADSCs
For the preparation and implantation of ADSCs, all animals were submitted to abdominal fat collection 2 weeks before the experimental lesion. To collect fat, the animals were anesthetized with the same protocol, and then a lower abdominal median incision was performed, with dissection by planes until the aponeurosis of the rectum muscle. Identification of the left superficial epigastric artery in the inguinal region was performed, and a fat fragment with weight ranging from 2 ± 0.5 grams was collected. The fat fragment was transported, in PBS buffer solution, from the collection site to the laboratory to follow the specific procedures of preparation of ADSCs.

Preparation of ADSCs
The preparation of the cells followed a protocol that had been previously published. Briefly, the fat was washed extensively with phosphate buffer saline (PBS), minced, and enzymatically digested at 37 °C for approximately 30 min using 0.075% collagenase type IA (Sigma; St. Louis, MO, USA). The digestion was performed with continuous agitation, with periodic changes in the solution, until the fragments were homogenized. The homogenization was assessed by light microscopy, and the digestion process was allowed to continue until the cells were adequately dissociated, which was approximately 3 hours, according to the manufacturer’s instructions. The digestion was stopped by adding the neutralizing agent, in amounts sufficient to neutralize the enzyme activity. The cells were washed three times with PBS, and the supernatants were discarded.

Fig. 1 General experimental design. Description: The image represents the general division of the groups from the first stage of the procedure, when the collection of fat took place, to the respective evaluations.

Fig. 2 Experimental model of muscle injury. Description: (A) Exposure of the recto femoral muscle to its full extent. (B) Experimental lesion in the middle third of the femoral rectus muscle. (C) Marking the extremities of the femoral rectus muscle injury with non-absorbable point.
The cellular confluence was avoided to prevent potential spontaneous differentiation. Culture media was changed every 23 days. Cells were rinsed with PBS and incubated with 1:100 dilution of dialkylcarbocyanine solution, a fluorescent cell membrane marker, (Vybrant Dil; Molecular Probes, Eugene, OR) for 30 min at 37 °C in accordance with the manufacturer’s protocol. The labeled cells were harvested with 0.25% trypsin/1 mM EDTA solution. To perform the autologous transplantation, cells were suspended to a concentration of 1-106 of labeled cells.

ADSCs Implant
The paws included in group III—ADSCs were initially submitted to the protocol of experimental muscle injury and then submitted to the application of ADSCs directly on the site of the lesion.15 The application occurred through direct visualization with intramuscular infiltration of the 1-2 \times 10^6 of marked ADSCs.

Muscle Tissue Collection
After the 2-week postintervention period, the animals were anesthetized and then submitted to painless death through overdose of anesthetics (ketamine 200 mg/kg + xylazine 40 mg/kg and tramadol 10 mg/kg). For collection, a cutaneous incision was performed according to the previous route and division by planes until exposure of the previously injured region in the femoral recto muscle (previously marked with nylon 6-0). Then, the femoral recto muscle incision was performed in the region between the 6-0 nylon points (site of muscle injury). The collected material was stored in a formaldehyde solution at 4 °C to follow the entire histological evaluation protocol.

Histological Analysis
Material Preparation
The muscle fragments were fixed in 10% formaldehyde for 24 hours and dehydrated in increasing concentrations of ethyl alcohol, diaphanized by xylol and impregnated by liquid parafin in a greenhouse, regulated at 60 °C. In sequence, the blocks were cut into minot microtome, adjusted to 4 μm with a 50 μm distance between the cuts. The cuts thus obtained were placed on slides previously greased with Mayer albumin and kept in a regulated oven at 37 °C, for 24 hours, for drying and gluing. After preparation, the slides were stained with hematoxylin and eosin (H&E) and picrosirius red techniques.

Quantitative Evaluation of inflammatory process
In view of the existence of inflammatory processes resulting from tissue lesions, five images of each slide were obtained through an Olympus IX 81 optical microscope (Olympus Corporation, Shinjuku-ku, Tokyo, Japan) coupled to an Olympus DP72 camera (Olympus Corporation, Shinjuku-ku, Tokyo, Japan). These images, obtained with an increase of 40X, were analyzed with the help of ImageJ Software (ImageJ 1.53h, National Institutes of Health, Bethesda MA, USA).

For analysis, the cells related to the scar inflammatory process were isolated through the plugin segmentation, thus excluding the nuclei referring to muscle fibers, and then the plugin counter cell was applied to quantify the number of total cells remaining in each image. The data obtained were compiled and later separated between the groups (Group II—SHAN or Group III—ADSCs). At the end, a comparative analysis was performed regarding the effects of treatment with ADSCs on muscle healing.

Qualitative Assessment of Muscle Healing
Considering the healing process of muscle injury and collagen changes that occur over time, a qualitative methodology was performed using as reference the muscle healing process and the respective color changes over this period. For this analysis, we used the images obtained through the microscope Zeiss AX10 (Zeiss, Jena, Thuringia, Germany) coupled to a Zeiss AxioCam ICC5 camera (Zeiss, Jena, Thuringia, Germany) of the slides colored in red picrosirius. In a simplified way, a descriptive analysis was performed on the proportion of fibers in an advanced healing aspect, that is, with yellow/orange coloration.

Macrosopic Analysis
The evaluation of the local morphology was performed at the immediate moment of material collection. In this evaluation, the following aspects were analyzed: changes in color, solidity, level of fibrosis, presence of infectious signs, and local inflammatory response.22 Additionally the images were also documented through Canon photographs (Canon EOS Rebel T5; Canon, Manaus, AM, Brazil) for further verification and presentation of results.

Sample Calculation and Statistical Analysis
Considering the pioneering of the study, the number of animals was decided after analysis of the relevant literature.15,23 The data obtained in the quantitative analysis of the inflammatory response were tabled and statistically analyzed with the BioStat 2009 program (AnalystSoft Inc., Alexandria, VA, USA). First, the data were submitted to the Shapiro-Wilk test to verify the normality of the groups and after the analysis of variance (ANOVA)/Tukey test for parametric data, and Kruskal-Wallis/Dunn for non-parametric data, to determine the significance of the results. The level for rejection of the null hypothesis was set at 5% (p ≤ 0.05), with an asterisk marking the significant values.
Results

Histological Analysis

The inflammatory process of muscle healing was ongoing in both groups, given the presence of inflammatory tissue in the sample analyzed. However, the quantitative analysis showed that the addition of ADSCs is related to a decrease in the number of inflammatory cells per field in the 2-week evaluation (►Figure 3). On the quantitative analysis, we noticed a decrease from 164.2 cells in the group without the addition of ADSCs to 89.62 cells per field in the group with the addition of ADSCs, representing a 46% decrease in the number of inflammatory cells after the addition of ADSCs (►Figure 4). Considering that the control group did not present any evidence of inflammatory process, it did not enter this quantification.

The picrosirius red technique, under polarization, showed that the treated group had more orange/yellow fibers, which evidences accumulation of thicker collagen fibers compatible with the final healing process (►Figure 5).

Macroscopic Analysis

There was no change in the general state of the animal or infectious signs in the hind legs submitted to experimental injury or intervention with the addition of ADSCs. The animals were able to walk in the cage in the first postoperative days and at no time presented modification in the acceptance of the diet or water. In both groups, the animals’ muscle tissue already presented a healing aspect in the final phase, with remarkable fibrotic changes on its surface. The macroscopic evaluation did not show any differences between the group submitted and the one not submitted to intervention (►Figure 6).

Discussion

The main findings of the present study refer to the achievement of good histological results after the use of ADSCs in the treatment of acute muscle injury. These findings are added to recent studies published by our group, which show promising results for the use of different orthobiologics, (i) stem cells and (ii) scaffolds. These first studies were able to satisfactorily reproduce well-established results in other research groups and are functioning as motivators for continuity in the development, improvement, and use of orthobiologics.

In the experimental model presented, with an acute, cutting lesion of muscle tissue, we hope that the use of ADSCs will be able to optimize muscle healing through three mechanisms, (i) production of growth factors, with optimization of angiogenesis and reduction of pathways that favor cellular apoptosis; (ii) immunosuppressive
action by decreasing activity in T and B lymphocytes; and (iii) induction in the differentiation of fibroblasts into myocytes.

The choice of an acute injury with early evaluation was motivated by greater functionality and performance of stem cells in the first days after the intervention. Thus, we tried to evaluate a probable acceleration in the functional recovery over time, after the use of ADSCs. In this sense, the great innovation of this work was precisely to present the first study using ADSCs in the treatment of acute muscle injury in an experimental model.

Among the limitations of the present study, we can mention the difficulties for sample calculation, given the pioneering of the study; the use of an experimental model that is not reproducible in clinical practice, since the scathing lesions are not the most frequent; and the lack of additional evaluation with other methods, such as biomechanics and functional evaluation. As prospects for the future, we hope to maintain pioneering and continue the work with development, production, and evaluation of the most diverse orthobiologics available.

Conclusion

The use of ADSCs in the treatment of acute muscle injury showed histological advantages when compared to their non-use.

Financial Support

The study was funded by the National Council for Scientific and Technological Development (CNPq) - process number 311237/2018-5.

Authors’ Contributions

Each author contributed individually and significantly to the development of the present article.
Conflict of Interests
The authors declare that there is no conflict of interests.

References