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

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

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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.

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

* *Multicenter study developed in two research centers at Escola Paulista de Medicina, Universidade Federal de São Paulo and at the Department of Biological Sciences, Campus Diadema, UNIFESP, São Paulo, Brazil.*

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Resumo

Objetivo Avaliação histológica e macroscópica do processo de cicatrização das lesões agudas do músculo reto femoral, com utilização de células-tronco derivadas de tecido adiposo (ADSCs, na sigla em inglês).

Método Foi realizado um estudo experimental com 18 patas traseiras de coelhos Nova Zelândia, que foram divididos em três nos grupos de estudo de acordo com a intervenção a ser realizada. No grupo I não foi realizado procedimento cirúrgico; no grupo II – SHAN foi realizado a lesão experimental sem nenhum protocolo de intervenção adicional; e no grupo III – Intervenção foi realizado a adição de ADSCs na mesma topografia onde foi realizada a lesão experimental. Após o período proposto, 2 semanas, o material foi coletado, submetido a avaliação macroscópica e histológica.

Resultados A análise quantitativa demonstrou que a adição de ADSCs está relacionada com a diminuição de células inflamatórias na avaliação com 2 semanas (164,2 células no grupo II – SHAN para 89,62 células no grupo III – ADSC). A análise qualitativa das lâminas coradas com Picrosírius red demonstrou um aumento das fibras de cor laranja/amarela no grupo III – ADSC, o que evidencia um processo final de cicatrização. A avaliação macroscópica não encontrou diferença entre os grupos.

Conclusão A utilização de ADSCs no tratamento de lesão muscular aguda apresentou vantagens histológicas quando comparada a sua não utilização.

Palavras-chave

- células-tronco mesenquimais
- medicina regenerativa
- doenças musculares
- músculos
- regeneração

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 activities[3,8] motivates the constant search for new therapies that can improve the results. Seeking to fill this space, the use of orthobiologicals has been gaining space in the treatment of various orthopedic lesions, including muscle injuries.[9] Among the available orthobiologicals, the use of adult mesenchymas 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 histological 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

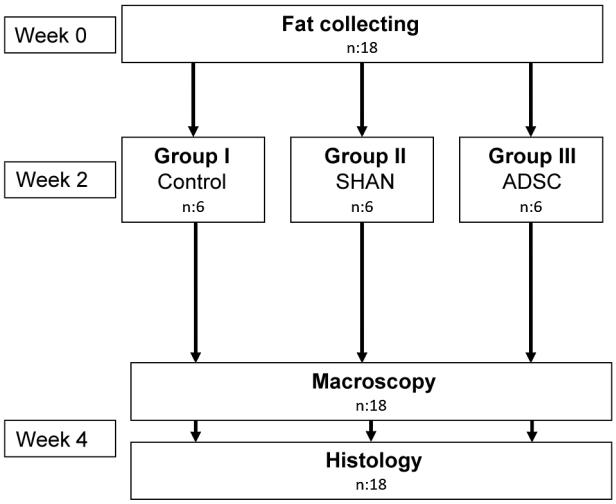

Fig. 1 General experimental design. Description: The image represents the general division of the groups from the first stage of the procedure, when the collection of fat took place, to the respective evaluations.

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, ketatamine (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, dilvulsion 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.[19,20] 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 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.[10,11,21]

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.[10,11,21] 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,

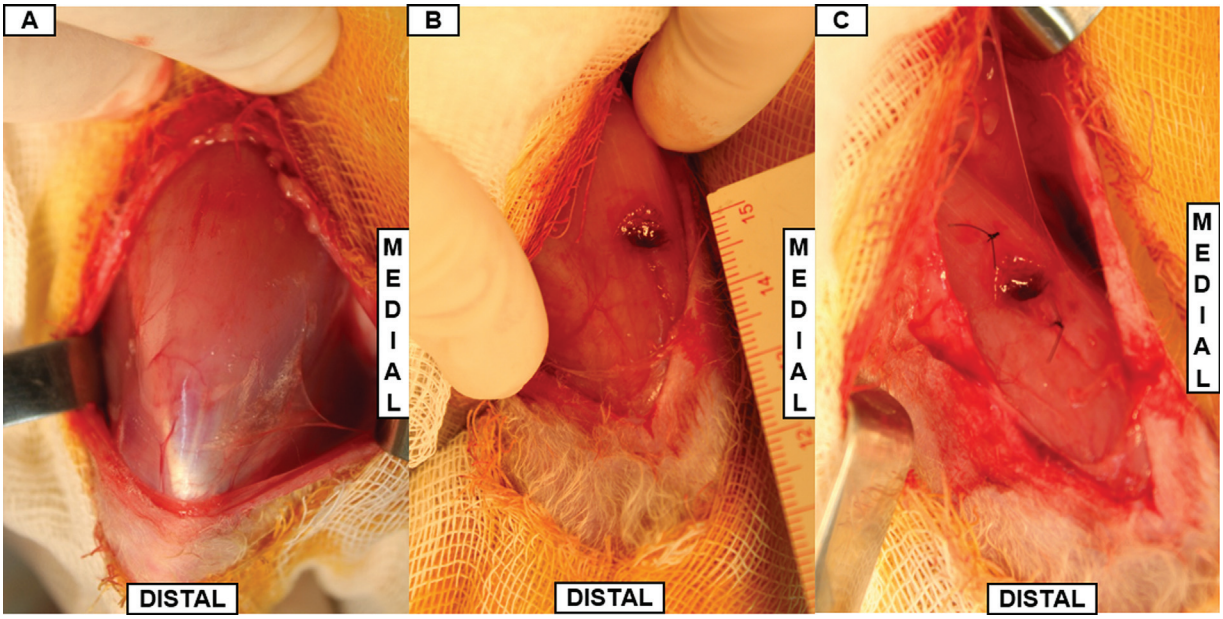

Fig. 2 Experimental model of muscle injury. Description: (**A**) Exposure of the recto femoral muscle to its full extent. (**B**) Experimental lesion in the middle third of the femoral rectus muscle. (**C**) Marking the extremities of the femoral rectus muscle injury with non-absorbable point.

MO). The digested tissue was filtered using 100 mm strain to obtain a cell suspension containing the stromal vascular fraction. After centrifugation, the pellet was resuspended in culture media (CM) consisting of Dulbecco's modified Eagle's media (DMEM; Mediatech, Herndon, VA), 10% fetal bovine serum (FBS Gibco; Grand Island, NY), and 1% of a solution of antibiotic/antimycotic (penicillin G 10,000 U/ml, amphotericin B 25 mg/ml and streptomycin 10,000 mg/ml). Cells were allowed to adhere to the flask for 24 h, after which fresh media was added. The cells were incubated at 37 °C and 5% CO_2 in CM until they reached semi-confluence. 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 DiI; 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/ 1mM EDTA solution. To perform the autologous transplantation, cells were suspended to a concentration of 1-2×10^6 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 1-2×10^6 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 divulsion 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 10% 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 paraffin 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.

Macroscopic 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% ($p \leq 0.05$), with an asterisk marking the significant values.

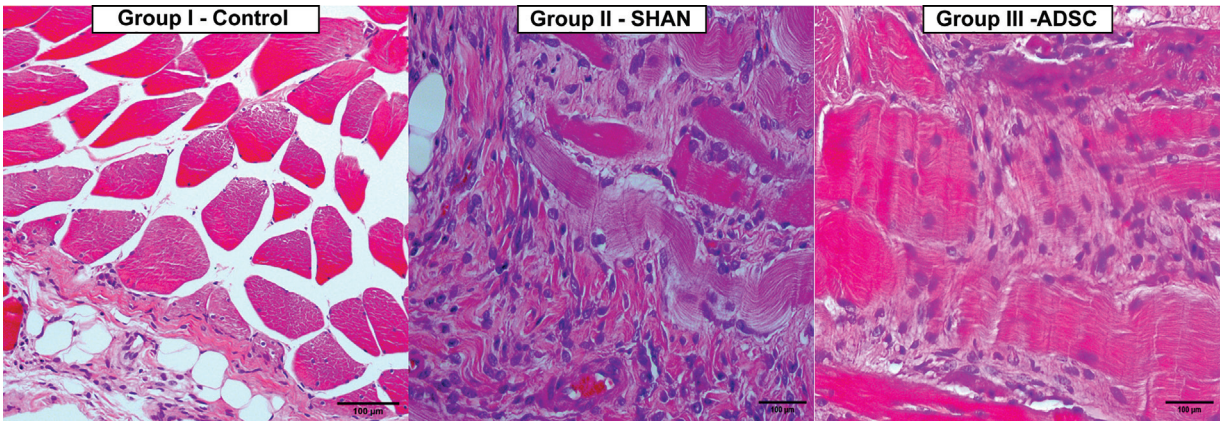

Fig. 3 Microscopic histology of muscle healing—Inflammatory evaluation. Description: Images representative of muscle healing in different groups. Group I—Control: Hematoxylin and eosin (H&E) image with an increase of 40X, representative of the original muscle tissue, not submitted to injury or intervention protocols, presenting muscle tissue with habitual appearance. Group II—SHAN: H&E image with an increase of 40X, representative of the group submitted only to experimental lesion with two weeks of evolution, presenting a large amount of inflammatory tissue and muscle tissue in the initial phase of healing. Group III—ADSCs: H&E image with an increase of 40X, representative of the group submitted to experimental injury and treatment with the use of ADSCs, showing a decrease in the number of inflammatory cells, and muscle tissue in the early process of healing.

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.

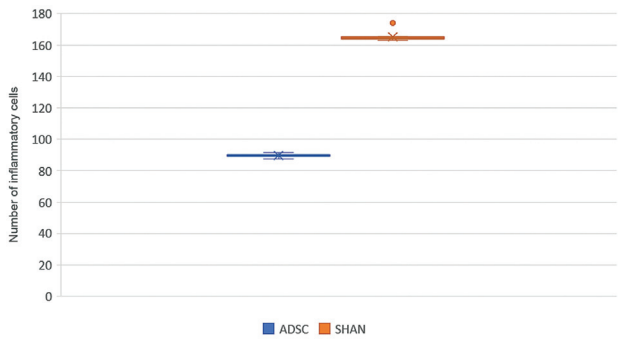

Fig. 4 Graphic image of inflammatory analysis. Description: Images representative of the average number of cells per group. In blue, the number of cells per field of the group submitted to treatment with ADSCs and, in orange, of the group not submitted to any treatment is represented. In this analysis, there is an important decrease in the number of inflammatory cells after the addition of ADSCs in the treatment of acute muscle injury.

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[15] and (ii) scaffolds.[22] 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,[24] (i) production of growth factors, with optimization of angiogenesis and reduction of pathways that favor cellular apoptosis; (ii) immunosuppressive

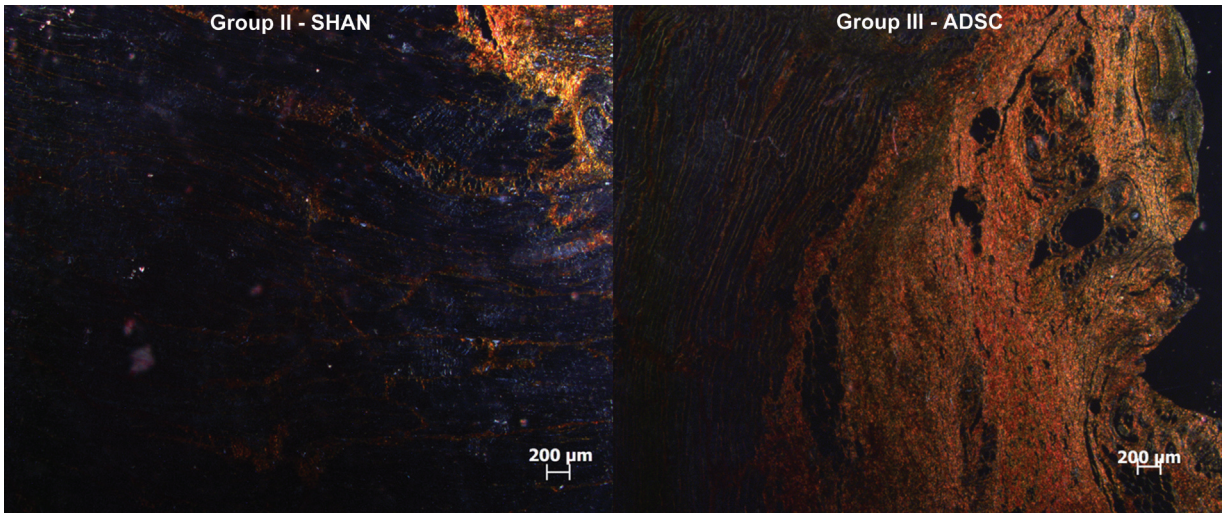

Fig. 5 Microscopic histology of muscle healing - muscle evaluation. Description: Images representing collagen modifications in groups submitted to experimental injury and treatment with ADSCs. Group II—SHAN: Picrosirius image, representative of the initial phase of collagen modification with minimal number of fibers colored in orange or yellow. Group III—ADSCs: Picrosirius image, representative of more advanced phase of collagen modification with a greater number of fibers colored in orange or yellow.

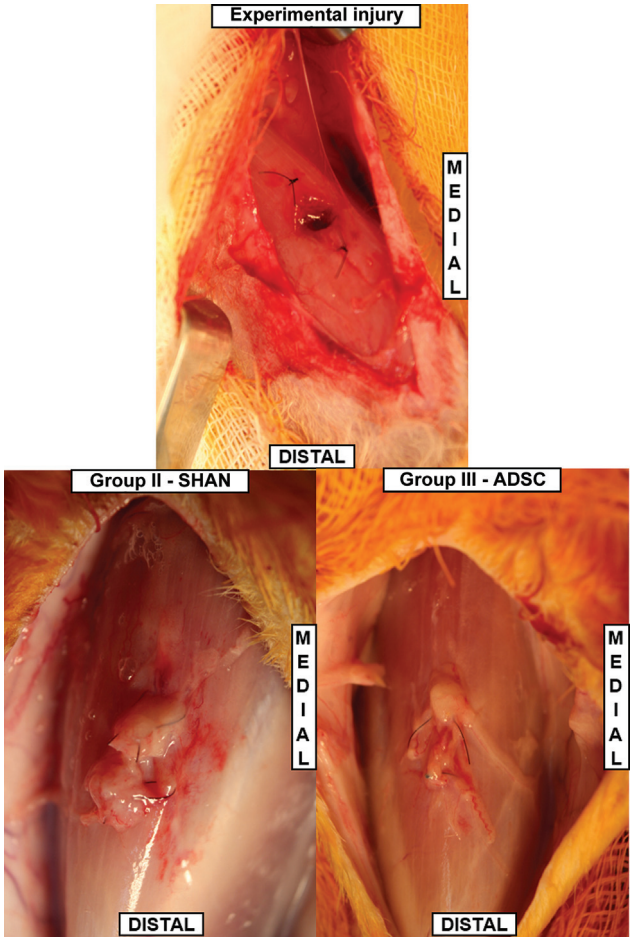

Fig. 6 Macroscopic evaluation using ADSCs. Description: Experimental injury - aspect of the experimental model immediately after muscle injury and marking with nylon 6-0. Group II—SHAN: Representative image of the macroscopic aspect after 2 weeks postoperatively. Group III—ADSC: Representative image of the macroscopic aspect of the group submitted to injury and intervention (addition of ADSCs) after 2 weeks postoperatively.

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.

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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.

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