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Equine Bone Marrow-Derived Mesenchymal Stem Cells - Evaluation of Muscle Inflammatory Response Following Intramuscular Transplantation

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ABSTRACT

***Background*:** Horses hold significant value in sports and the economy, requiring rapid recovery from musculoskeletal injuries. Cell-based therapies, particularly those using mesenchymal stem cells (MSCs), have emerged as promising tools due to their regenerative, anti-inflammatory, and immunomodulatory properties, as well as their capacity for self-renewal. However, their therapeutic efficacy may be influenced by host immune responses, particularly in allogeneic settings. Thus, this study aimed to morphologically evaluate the acute inflammatory response in equine muscle tissue following intramuscular transplantation of autologous and allogeneic bone marrow-derived MSCs (EqBM-MSCs).

***Materials, Method & Results*:** Fifteen clinically healthy adult horses (6-12 years; 300-500 kg) were randomly assigned to 3 groups (n = 5 each): Control (intramuscular injection of phosphate-buffered saline - PBS), Auto-MSCs (intramuscular transplantation of autologous EqBM-MSCs), and Allo-MSCs (intramuscular transplantation of allogeneic EqBM-MSCs). EqBM-MSCs were isolated from bone marrow, expanded under standard culture conditions, and characterized by plastic adherence, fibroblastoid morphology, expression of CD44 and CD90, absence of CD34, and their differentiation potential into adipogenic, osteogenic, and chondrogenic lineages. An average of 2×10^6 cells in 100 µL of PBS were transplanted into the right *gluteus medius* muscle under ultrasound guidance. Two muscle biopsies were collected per horse before transplantation (D0) and 24 h post-transplantation (D24). Histological hematoxylin and eosin (H&E); Gomori's trichrome (GT)) and histochemical (nicotinamide adenine dinucleotide tetrazolium reductase (NADH-tr)) analyses demonstrated preserved muscle architecture and normal fiber organization in both MSC-treated groups. No evidence of inflammation, such as fiber morphological alterations or inflammatory cell infiltration, was observed in any group.

***Discussion*:** MSCs possess self-renewal capacity and secrete bioactive factors that support tissue repair and modulate inflammation. In equines, bone marrow is a commonly used MSC source, and intramuscular delivery has been associated with efficient administration and prolonged local retention. The absence of acute muscle inflammatory changes observed in this study is consistent with previous reports in equine tendon following MSC transplantation. In contrast, inflammatory responses have been described in rat muscle after autologous bone marrow MSC administration, typically characterized by muscle fiber alterations and inflammatory cell infiltration. Such changes were not observed in the present study, and NADH-tr staining confirmed preserved metabolic activity. These findings indicate that intramuscular administration of both autologous and allogeneic EqBM-MSCs is well-tolerated and does not induce acute inflammation. The lack of acute response may be related to the immunomodulatory properties of MSCs, including secretion of factors such as transforming growth factor beta (TGF-β) and prostaglandin E2 (PGE2). The absence of inflammatory changes supports the immunomodulatory properties of MSCs and may reinforce their potential for clinical application in equine muscle injury. Nevertheless, the evaluation performed at 24 h reflects only the acute phase, and longer-term responses remain to be elucidated. Further studies are necessary to investigate the long-term safety and therapeutic potential of intramuscular MSC transplantation in equine musculoskeletal disorders.

Keywords: autologous, cell therapy, *gluteus medius*, histology, histochemistry.

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INTRODUCTION

Horses play a pivotal role in sports, holding significant economic importance, thereby emphasizing the need for rapid recovery from musculoskeletal injuries [3]. In recent years, cell-based therapies have emerged as promising tools to aid in the treatment of these injuries in equines [16].

MSCs have garnered considerable attention in cellular therapy for their ease of isolation, expansion capacity, and ability to differentiate into various cell lineages [1]. Moreover, MSCs exhibit therapeutic properties such as anti-inflammatory, antiapoptotic, immunomodulatory, and neuroprotective effects [12].

In equines, MSCs can be obtained from various tissues, with bone marrow-derived MSCs (BM-MSCs) being extensively studied [20]. However, the therapeutic efficacy of MSCs transplantation can be influenced by the immune response of the recipient [2].

Autologous therapy is hindered by the time required for cell isolation and expansion, limiting its application in acute injuries [14]. Conversely, allogeneic therapy offers the advantage of immediate availability and careful cell selection [17]. Nevertheless, a study in equines describe that intra-articular administration of allogeneic MSCs may provoke a more pronounced inflammatory response post-transplantation [15]. Allogeneic MSCs are no longer considered immune-privileged but rather immune-evasive, and they may still be recognized and eliminated by the host immune system [2].

Therefore, this study aims to morphologically evaluate the acute inflammatory response in equine muscle tissue following intramuscular autologous and allogeneic transplantation of equine BM-MSCs (EqBM-MSCs), contributing to the ongoing optimization of cell-based therapies for equine musculoskeletal injuries.

MATERIALS AND METHODS

Animal selection

For this study, 15 adult mixed-breed horses, including both females and males, aged between 6 and 12 years-old and weighing between 300 and 500 kg, were selected. The horses were fed twice daily with chopped grass, coast cross hay, and commercial concentrate. Water and mineral salt were provided *ad libitum*. During the experimental period, the horses were housed in paddocks at the Veterinary Teaching Hospital, School of Veterinary Medicine and Animal Science (FMVZ) of São Paulo State University (UNESP) in Botucatu, Brazil. All animals were dewormed, weighed, and underwent a physical examination prior to the experiment. Only clinically healthy animals were included in this study.

Experimental design

The animals were randomly assigned to 3 experimental groups. In the control group (Control, n=5), intramuscular (IM) phosphate-buffered saline (PBS)[1] was administered. The Allo-MSCs group (n=5) underwent IM transplantation of allogeneic EqBM-MSCs, while the Auto-MSCs group (n=5) received IM transplantation of autologous EqBM-MSCs.

Cell transplantation was carried out using ultrasound[2] guidance in the right *gluteus medius* muscle of the animals. To assess acute inflammatory response, 2 muscle biopsies were performed, 1 before transplantation (D0) and another 24 h after (D24). The samples underwent histological and histochemical analysis (Figure 1).

EqBM-MSCs isolation and characterization

Bone marrow aspiration was performed according to the methodology described previously [11]. The animals were sedated intravenously with 10% xylazine hydrochloride[3] (0.5 mg/kg). Then, a local anesthetic block was administered using 2% lidocaine hydrochloride[4] at the site of the 5th sternebra, where bone marrow cells were subsequently collected using a Komiyashiki® needle.

The bone marrow samples were initially filtered. Subsequently, the samples were centrifuged at 340 × *g* for 10 min, and the supernatant was discarded. To the remaining material, Dulbecco's Modified Eagle's Medium (DMEM) low glucose medium[5] was added in a 1:1 ratio. Histopaque-1077[6] was then added slowly (1:1) followed by centrifugation at 340 × *g* for 40 min. After centrifugation, the mononuclear cell fraction was collected and resuspended in DMEM low glucose/F12[5]. The suspension was centrifuged twice at 340 × *g* for 10 min.

The cells were cultured in complete medium composed of DMEM low glucose/F12 (1:1)[5], 20% fetal bovine serum[5], 1% penicillin/streptomycin[5], and 1.2% amphotericin B[5], at 37.5°C in a humidified atmosphere with 5% CO_2 and 95% air. The culture medium was replaced every 2 or 3 days until reaching 80% cell confluence.

The immunophenotypic characterization of MSCs was conducted at the end of primary culture using a FACS Calibur flow cytometer[7] with the following antibodies mouse anti-rat CD90-FITC (clone OX7)[8], mouse anti-human CD34-FITC (clone 581)[7], mouse anti-horse CD44 (clone CVS18)[9]. A secondary antibody, goat anti-mouse IgG-FITC[9], was used according to the manufacturers' protocols. During analysis, at least 10,000 events were counted.

To induce adipogenic, osteogenic, and chondrogenic differentiation, EqBM-MSCs were cultured in 12-well plates. After 48 h, the complete culture medium was removed and replaced with osteogenic, adipogenic, chondrogenic differentiation medium StemPro®[5]. The medium was changed every 3 days. Adipogenic differentiation was confirmed by the presence of intracytoplasmic lipid droplets, identified using Oil Red stain[6]. Osteogenic differentiation was confirmed by mineralized matrix deposition, as demonstrated by positive Alizarin Red staining. Chondrogenic differentiation was confirmed by the identification of an extracellular matrix rich in proteoglycans, evidenced by Alcian Blue-positive staining.

EqBM-MSCs transplantation

An average 2 x 10^6 EqBM-MSCs suspended in PBS1 were used for cellular transplantation into the right *gluteus medius* muscle of the animals. In the control group, 100 µL of PBS was administered; in the Auto-MSCs group, 100 µL of autologous EqBM-MSCs; and in the Allo-MSCs group, 100 µL of allogeneic EqBM-MSCs

Cell transplantation was guided by ultrasound[2] to determine the site and depth of the procedure. The application was performed using a 40×12 mm hypodermic needle and a 1 mL syringe. The site and depth were marked by changes in echogenicity observed at the cell injection site compared to adjacent muscle fibers (Figure 2).

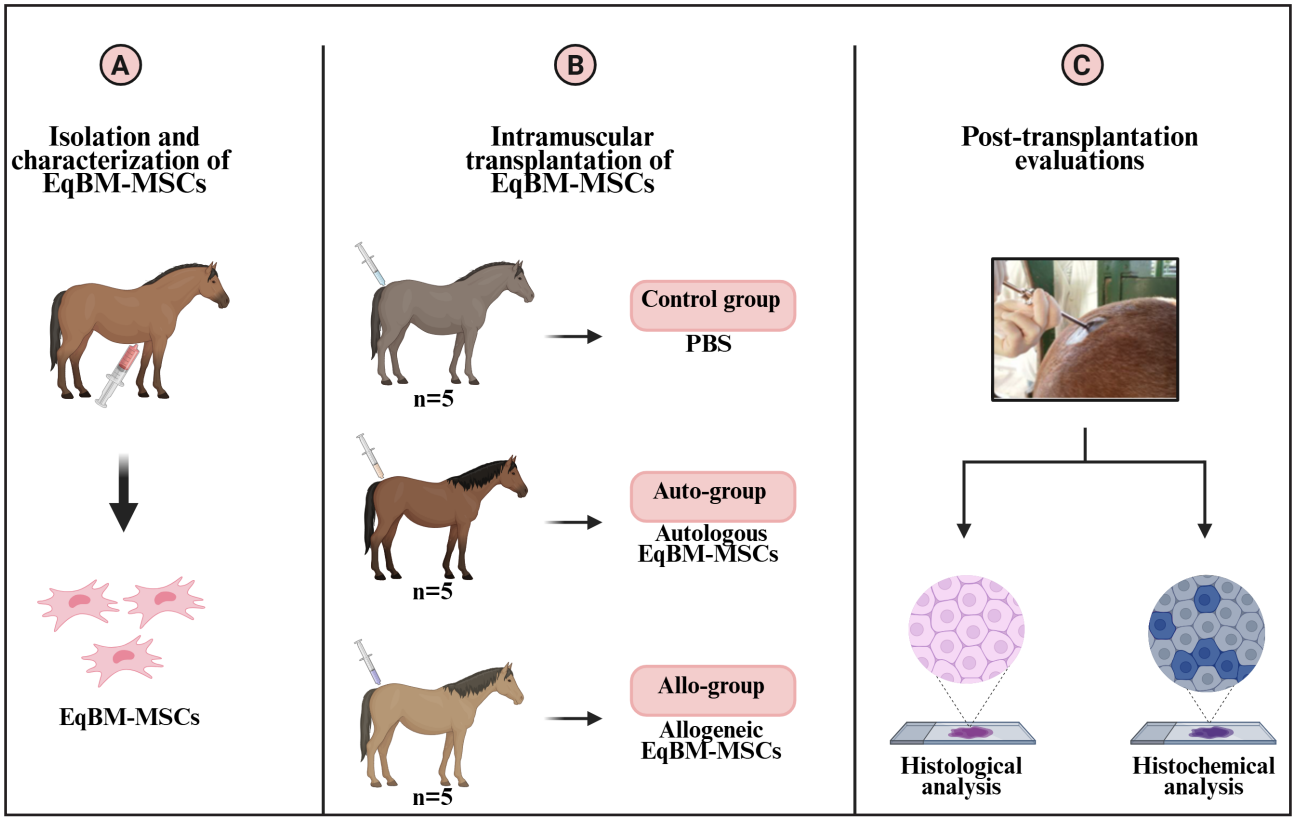

Figure 1. Experimental design overview. A- Bone marrow aspirate was used to isolate and characterize equine bone marrow–derived mesenchymal stem cells (EqBM-MSCs). B- Fifteen animals were randomly assigned to 3 groups. Control group (n=5), receiving intramuscular (IM) phosphate-buffered saline (PBS), Allo-MSCs (n=5), undergoing IM transplantation of allogeneic EqBM-MSCs, and Auto-MSCs (n=5), receiving IM transplantation of autologous EqBM-MSCs. Transplantation was performed in the right *gluteus medius* muscle. C- Acute inflammatory response was evaluated through two muscle biopsies, one before transplantation and another 24 h after, followed by histological and histochemical analysis of the samples. [Created in BioRender. Amorim, R. (2026) https://BioRender.com/0h9lzj3].

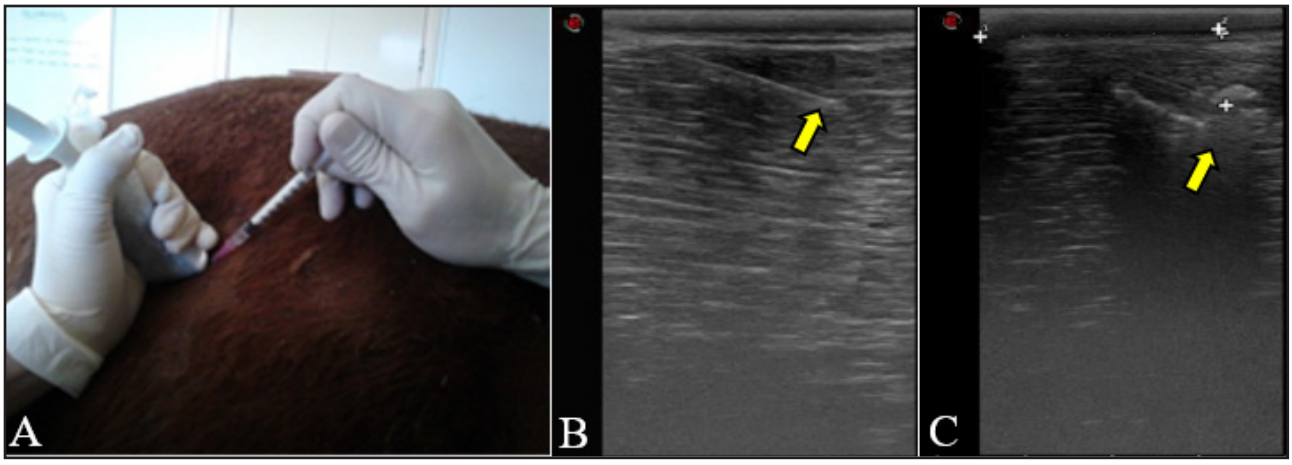

Figure 2. Intramuscular transplantation of equine bone marrow-derived mesenchymal stem cells (EqBM-MSCs). A- Ultrasound-guided application. B- Ultrasound image showing the needle penetrating the muscle (yellow arrow). C- EqBM-MSCs transplantation, highlighting a hyperechoic area compared to adjacent muscle fibers (yellow arrow).

Muscle biopsies

Two muscle biopsies were performed on the right *gluteus medius* muscle of the horses in the present study. The 1st biopsy was conducted 1 day before the cell transplantation procedure (D0), and the 2nd biopsy was performed 24 h after the cell transplantation (D24), adjacent to the site of the 1st biopsy. The biopsies were conducted to harvest tissue samples from the area where the cells were implanted. The muscle samples were then frozen and evaluated using histological and histochemical techniques.

The biopsies were performed on the *gluteus medius* muscle using a 6.0 Bergström needle (6 mm). The obtained samples were placed in a Petri dish covered with gauze soaked in physiological solution to halt any muscle contraction processes. They were then coated with neutral talcum powder to promote cryoprotection, preserving muscular architecture during freezing and storage in liquid nitrogen. Subsequently, the muscle fragment was sectioned using a cryostat at -20°C and analyzed using histological and histochemical staining techniques.

Histological and histochemical analysis

To assess tissue architecture, Hematoxylin & Eosin (H&E) and Gomori's Trichrome (GT) staining, were performed. These stains allowed for the analysis of muscle fiber morphology and non-contractile elements such as nerves, blood vessels, connective tissue, adipose tissue, and nuclei [6]. Evaluation criteria included increased vessel count, fiber disarray, and heightened cellularity.

For histochemical analysis, nicotinamide adenine dinucleotide tetrazolium reductase (NADH-tr) staining was used to evaluate the oxidative capacity of muscle fibers. The slides were digitized for microscopic analysis using Leica Qwin software, allowing differentiation of the 3 types of muscle fibers (I, IIA, and IIX).

RESULTS

EqBM-MSCs exhibited mesenchymal fate

The EqBM-MSCs exhibited fibroblastoid morphology (Figure 3A), adherence to plastic, and differentiation into trilineage *in vitro* confirming their multipotentiality.

Fat droplets observed in the cytoplasm confirmed adipogenic differentiation, evident from Oil Red staining (Figure 3B). Osteogenic differentiation was demonstrated by mineralized matrix deposition, as evidenced by positive Alizarin Red staining (Figure 3C). Chondrogenic differentiation was confirmed by the presence of an extracellular matrix rich in proteoglycans, demonstrated by positive staining with alcian blue (Figure 3D).

The immunophenotyping analysis showed expression of the CD44 (means = 81.68% and 92.10%) and CD90 (means = 87.72% and 81.03%) markers in autologous and allogeneic MSCs, respectively, and absence of CD34 marker expression in both.

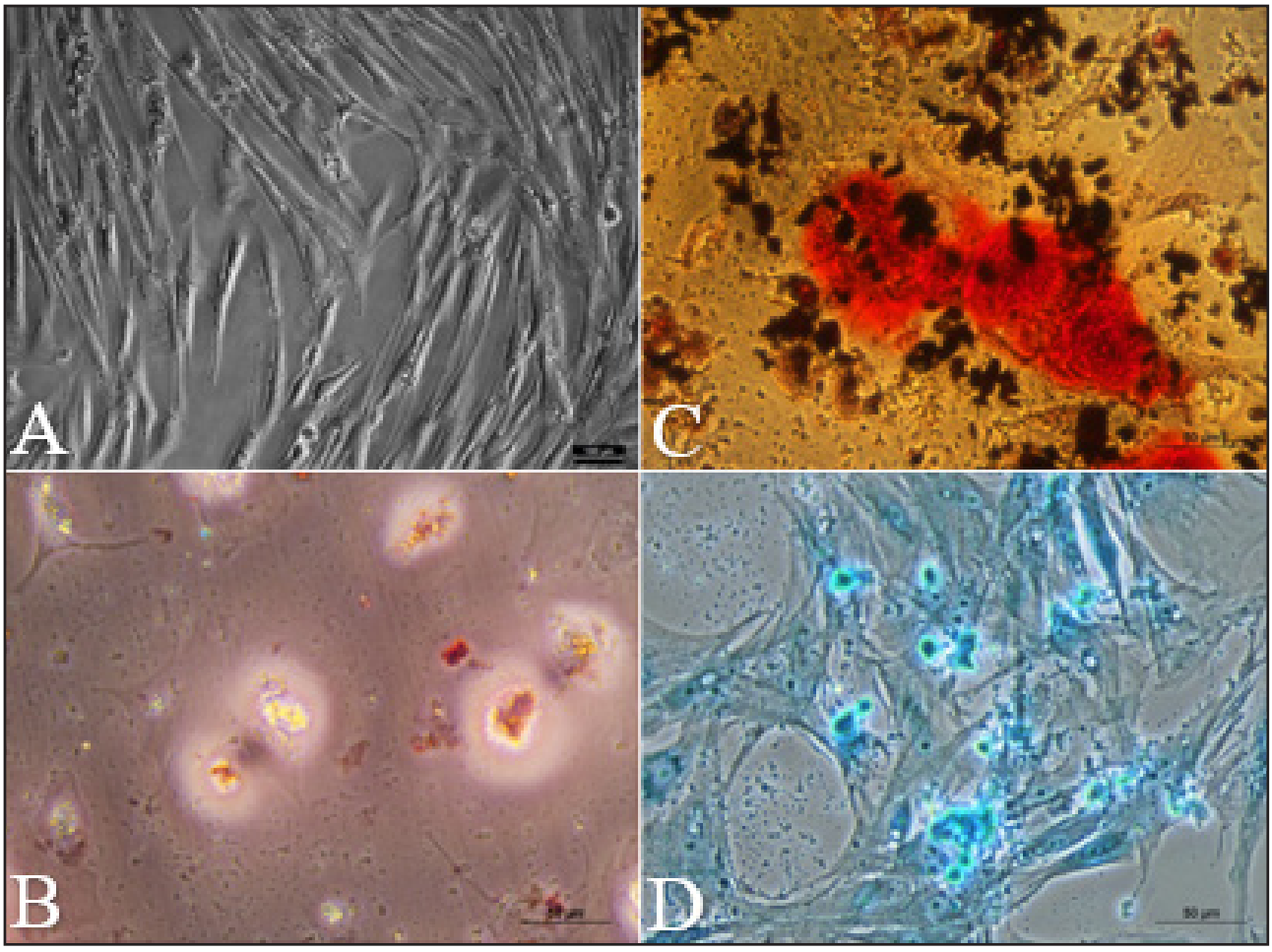

Figure 3. Characterization of equine bone marrow-derived mesenchymal stem cells (EqBM-MSCs). A- Fibroblast-like morphology of EqBM-MSCs. [Scale bar =100 μm]. B- Adipogenic differentiation showing lipid droplets, stained with Oil Red O. C- Osteogenic differentiation was evidenced by extracellular matrix mineralization, as demonstrated by positive Alizarin Red staining. D- Chondrogenic differentiation was confirmed by Alcian Blue staining, which revealed an extracellular matrix rich in proteoglycans. [Scale bar = 50 μm].

EqBM-MSCs transplantation preserves muscle tissue morphology

Histological and histochemical evaluation of the muscle tissue before transplantation is depicted in Figure 4. The analyses revealed that morphological integrity was preserved in the groups following EqBM-MSCs transplantation (Figure 4). Typical features of muscle inflammation, such as changes in muscle fiber shape and size, and infiltration of inflammatory cells, were not observed in any of the groups.

DISCUSSION

Musculoskeletal lesions often result in reduced performance or even premature retirement for equines [4,16]. To address these challenges, cell-based therapies offer promising avenues for treating these conditions in these animals [5]. MSCs have been widely used due to their self-renewal capacity, potential for cell differentiation, production, and secretion of various bioactive factors for regeneration of damaged tissue [12,19].

Cell transplantation routes can influence therapeutic success, and several approaches are being investigated, with emphasis on the intramuscular route in rats and mice for its efficient delivery and prolonged cell retention time at the site [9,10]. In a study involving horses, it was demonstrated that transplanting MSCs derived from the umbilical cord into the superficial gluteal muscle was safe and showed promising clinical potential [5]. However, to our knowledge, there are no studies evaluating the acute inflammatory response after EqBM-MSC transplantation in the *gluteus medius* muscle in horses.

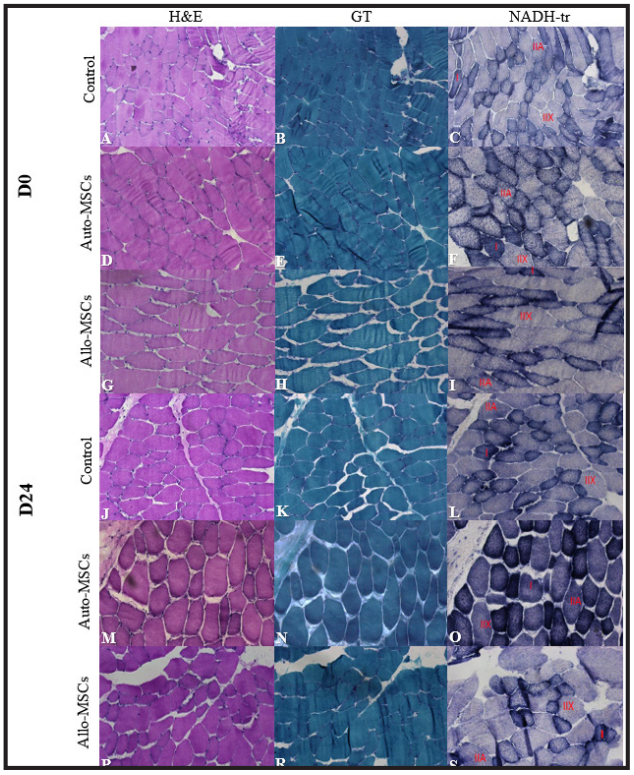

Figure 4. Histological and histochemical evaluation of the *gluteus medius* muscle in the 3 groups of this study before (D0) and 24 h after (D24) transplantation of equine bone marrow-derived mesenchymal stem cells (EqBM-MSCs). A-I: Before EqBM-MSCs transplantation. A-C: Control Group: A- Hematoxylin & Eosin (H&E) staining. B- Gomori's Trichrome (GT) staining. C- NADH-tetrazolium reductase (NADH-tr) staining (Type I, IIA, and IIX fibers). D-F: Auto-MSCs group: D-H&E staining. E- GT staining. F- NADH-tr staining (Type I, IIA, and IIX fibers). G-I: Allo-MSCs group: G- H&E staining. H- GT staining. I- NADH-tr staining (Type I, IIA, and IIX fibers). J-S: No changes were observed between the groups after EqBM-MSCs transplantation. J-L: Control Group: J- Hematoxylin & Eosin (H&E) staining. K- Gomori's Trichrome (GT) staining. L- NADH-tetrazolium reductase (NADH-tr) staining (Type I, IIA, and IIX fibers). M-O: Auto-MSCs group: M- H&E staining. N- GT staining. O- NADH-tr staining (Type I, IIA, and IIX fibers). P-S: Allo-MSCs group: P- H&E staining. R- GT staining. S- NADH-tr staining (Type I, IIA, and IIX fibers).

In histological and histochemical analyses of the *gluteus medius* muscle, we did not observe features of acute inflammation following EqBM-MSC transplantation in any of the groups. Similar results have been previously described following cell transplantation in equine tendon tissue, where no differences were found in histological analysis between autologous and allogeneic administrations [8]. However, a study has reported the development of an acute inflammatory response following autologous BM-MSC transplantation in rat muscle tissue [7]. The inflammatory process typically involves changes in muscle fiber size, and the presence of inflammatory cells at the site [18], none of which were observed in the groups of this study. Additionally, no alterations were detected in NADH-tr staining, indicating no impairment in oxidative fiber metabolism following MSC administration. The absence of such changes suggests that the transplantation of autologous and allogeneic EqBM-MSCs did not trigger a significant local inflammatory response within a 24-h period in the present study.

Besides their regenerative properties, MSCs are recognized for their immunomodulatory and anti-inflammatory capabilities due to the secretion of several factors such as transforming growth factor beta (TGF-β) and prostaglandin E2 [13]. These characteristics may explain the absence of inflammatory response observed after intramuscular transplants of MSCs, offering promising prospects for clinical applications in horses.

A limitation of this study is the lack of long-term evaluation of the safety of intramuscular transplantation in horses.

CONCLUSIONS

Intramuscular transplantation of allogeneic and autologous EqBM-MSCs into the *gluteus medius* muscle did not result in histological or histochemical changes after 24 h. Future studies are needed to assess the long-term safety of intramuscular transplantation in equines and its effectiveness in treating various types of injuries.

MANUFACTURES

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***Ethical approval*.** All phases of this study were approval from the Ethics Committee on Animal Use of UNESP - Botucatu. According to protocol number 44/2010. All procedures were carried out in accordance with international guidelines for the care and use of experimental animals.

***Declaration of interest*.** The authors have no relevant financial or non-financial interests to disclose. The authors alone are responsible for the content and writing of the paper.

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