UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

AVALIAÇÃO DA REAÇÃO INFLAMATÓRIA APÓS IMPLANTE SERIADO DE CÉLULAS TRONCO MESENQUIMAIS ALOGÊNICAS EM EQUINOS

MARINA LANDIM E ALVARENGA

BOTUCATU – SP
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MARINA LANDIM E ALVARENGA

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“ ...Quero permear-me com o poder da minha vontade e quero sentir como a serenidade se derrama por todo o meu ser quando eu me fortaleço para encontrar em mim a serenidade, como força pelo poder do meu esforço. ”

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Resumo

A utilização de terapias celulares no tratamento de lesões musculoesqueléticas cresce mundialmente em larga escala e é cada vez mais aceita. As células tronco mesenquimais (CTMs) são facilmente isoladas e expandidas *in vitro*, sendo tipicamente obtidas do próprio paciente (autóloga). Contudo, o uso de células provenientes de um animal doador (alogênica) e submetidas ao cultivo e a criopreservação, cria a oportunidade de iniciar o tratamento imediatamente após o diagnóstico. Há evidencias de que as CTMs possuem capacidade de imunomodulação, interagindo com os linfócitos T de diversas maneiras, além de não estimularem a indução de uma resposta imune complexa por não expressarem MHC II. Contudo, ainda há falta de consenso entre os estudos quanto a imunogenicidade das CTMs. Desta forma, o presente estudo objetivou avaliar a segurança da administração das células alogênicas em equinos através da análise de uma possível inflamação decorrente do implante alogênico de CTMs em músculo hígido de equinos, por meio de exame físico, ultrassonográfico, termográfico, histopatológico e pela expressão gênica de IL-1 beta e TNF- alfa. Os resultados demostraram que aplicação de CTM alogênicas é um processo seguro uma vez que não houveram alterações clínicas significativas, contudo uma segunda aplicação de CTM alogênicas de um mesmo doador levou ao aumento significativo da expressão gênica de TNF- alfa, podendo caracterizar uma resposta imune celular mais acentuada. Nossos resultados contribuíram para pesquisas futuras sobre tratamento de lesões musculoesqueléticas em equinos com CTMs, através da criação de bancos de células oriundas de doadores saudáveis.

Palavras-chave: equino, célula tronco mesenquimal, rejeição, músculo, inflamação

Abstract

The use of cell therapy for treatment of musculoskeletal injuries in horses is increasing in large scale worldwide. The mesenchymal stem cells (MSCs) are easily isolated and expanded in vitro, making them promising for use in clinical trials. Typically, these cells are obtained from the own patient (autologous), however, cell from a donor horse (allogeneic) expanded in vitro and cryopreserved create the opportunity of immediate treatment after diagnosis. There is evidence that MSCs have immunomodulatory capacity by interacting with T lymphocytes in several ways, and do not stimulate a complex immune response for not expressing MHC II. However, there is still a lack of consensus among the studies on the immunogenicity of MSCs. Thus, this study aimed to evaluate the safety of administration of allogeneic mesenchymal stem cells in horses. For such a possible inflammation reaction to the implantation was analysed through physical examination, ultrasound, thermography, histopathology and gene expression of IL 1beta and TNF-alpha. The results showed that the implantation of allogeneic MSCs is a safe process since there were no significant clinical changes in the animals, but a second application of allogeneic MSCs from the same donor led to significant increase in gene expression of TNF-alpha, which can characterize a cellular immune response. Our results contribute to future research on treatment of musculoskeletal injuries in horses using MSCs, by creating banks of cells derived from healthy donors.

Key-words: equine, mesenchymal stem cell, rejection, muscle, inflammation

CAPITULO 1

INTRODUÇÃO E JUSTIFICATIVA

As lesões do sistema musculoesquelético como miosites, tendinites, desmites, osteoatrites e laminite são muito frequentes em equinos, especialmente nos animais atletas. Estas lesões resultam na redução da vida útil ou até mesmo no encerramento da carreira atlética do animal, uma vez que a recuperação é dependente de um longo período de repouso e mesmo com o tratamento adequado a taxa de recidiva pode ser muito alta. Nesse sentido, a utilização de terapias com células tronco mesenquimais (CTMs), visando a regeneração tecidual, para o tratamento de lesões musculoesqueléticas em equinos cresce mundialmente em larga escala e é cada vez mais aceita.

As CTMs são facilmente isoladas e expandidas *in vitro*, sendo alvos promissores para aplicação em ensaios clínicos, particularmente objetivando a medicina regenerativa, terapia celular com imunomodulação e engenharia de tecidos.

As CTMs podem ser obtidas do próprio paciente (autóloga), contudo, a utilização de CTMs provenientes de um animal doador (alogênica) e submetidas ao cultivo e a criopreservação cria a oportunidade de iniciar o tratamento logo após o diagnóstico e oferece uma população celular maior e mais homogênea em um curto espaço de tempo.

Os estudos sobre terapia celular em equinos buscam a compreensão da biologia e das enfermidades musculoesqueléticas, permitindo analisar a segurança e a eficácia das técnicas, antes de sua aplicação em estudos clínicos. Desta forma, o presente estudo é um teste pré-clínico para tratamento de lesões músculoesqueléticas de equinos com as CTMs alogênicas, através da criação de bancos de células oriundas de doadores saudáveis. Baseados nesses resultados, o implante de CTMs alogênicas evitaria o tempo de espera entre a coleta do tecido adiposo ou medula óssea e o tratamento (tempo de cultivo para o implante autólogo), o qual muitas vezes é prejudicial para o paciente.

Um número limitado de cavalos com lesões de tecidos moles já foi tratado experimentalmente com CTMs alogênicas provenientes de medula óssea sem efeitos clínicos prejudiciais, sugerindo que este tipo celular possa ser utilizado no futuro próximo para o tratamento de lesões agudas logo após o seu

diagnóstico, superando dificuldades, como o tempo de cultivo prolongado. Todavia, a segurança e a ausência de resposta imune aguda ou tardia após múltiplas aplicações de CTM alogênicas em equinos ainda não foram claramente demostradas. Além disto, apesar de haver um número razoável de dados demostrando a baixa imunogenicidade e os efeitos imunomodulatórios sobre a ação das CTMs *in vitro*, pouca informação é fornecida sobre estas propriedades *in vivo*.

Devida aos poucos trabalhos presentes na literatura com o implante alogênico de CTMs em pacientes humanos e principalmente na medicina equina, acreditamos que este trabalho poderá contribuir para o melhor entendimento do comportamento local das CTMs alogênica, possibilitando assim diversas outras abordagens terapêuticas e laboratoriais. Sendo assim, o principal desafio científico deste projeto foi avaliar os efeitos do implante de células tronco mesenquimais alogênicas derivadas da medula óssea (MoCTMs) em tecido muscular hígido com intuito de qualificar e quantificar uma possível reação inflamatória local através de marcadores inflamatórios e parâmetros clínicos e ultrassonográficos. Dessa forma podemos avaliar a relevância clínica dessa reação e validar a possibilidade de utilização desse tipo celular na rotina clínica.

REVISÃO DE LITERATURA

As células-tronco (CT) podem ser definidas como células com grande capacidade de proliferação e auto renovação, além de serem capazes de responder a estímulos externos e dar origem a diferentes linhagens celulares. Estas células podem ser classificadas quanto seu grau de potencialidade como totipotentes, pluripotentes, multipotentes e unipotentes, e quanto a sua origem como embrionárias (CTE), adultas (CTA) (Zago e Covas, 2006; Migroni-Netto e Dessen, 2006) ou mais recentemente células tronco pluripotentes induzida (iPS) (Takahashi et.al.,2007).

Somente as células do embrião até a fase de mórula são totipotentes, ou seja, tem a capacidade de se diferenciar em todos os tecidos, incluindo a placenta. As CT pluripotentes podem se diferenciar nas três camadas clássicas do embrião, mas não em todos os anexos fetais. Já as células tronco adultas, ou

multipotentes só podem se diferenciar em linhagens especificas de células. Enquanto as unipotentes só de diferenciam em um único tipo celular (Avasthi et. al., 2008).

Dentre os diversos tipos de CT adultas estão as CT hematopoiéticas (CTH), conhecidas por dar origem a todos os tipos de células sanguíneas, tanto da linhagem mielóide como eritróide; e as CT mesenquimais (CTMs), as quais são células adultas indiferenciadas de origem mesodermal que podem ser isoladas de diferentes tecidos como medula óssea e tecido adiposo (Avasthi et. al., 2008).

As CTMs são caracterizadas pela sua capacidade de aderência ao plástico, pela expressão dos marcadores de superfície CD44, CD73, CD90 e CD105, entre outros, e pela não expressão de CD45, CD34, CD14 (CD11b), CD79 (CD19) e HLA- DR. Estas células podem dar origem a diversos tipos de tecidos estromais, podendo se diferenciar em células do tecido muscular, ósseo, adiposo, cartilaginoso e tendíneo (Koch et al, 2009; Ichim et al. 2010; Borjesson & Peroni, 2011). Apesar de originalmente terem sido isoladas da medula óssea, atualmente as CTMs já foram extraídas de diversos tecidos incluindo gordura, miocárdio, geléia de Wharton, polpa do dente, sangue periférico e cordão umbilical (Ichim et al., 2010; Lovati et al., 2010).

Os dois tipos celulares mais utilizados clinicamente em medicina veterinária são CTMs derivadas da medula óssea e do tecido adiposo. Em equinos a coleta da medula óssea é geralmente realizada do esterno ou da tuberosidade do íleo, e é de fácil execução quando comparado a outras espécies, uma vez que pode ser realizada com o animal em estação, sob sedação, sendo um procedimento simples, rápido e indolor, quando realizado adequadamente. Após a coleta o aspirado da medula óssea pode ser processado e submetido ao cultivo celular por 2-3 semanas para obtenção e expansão das CTMs; ou simplesmente submetido a centrifugação para a obtenção do concentrado de medula óssea. Ambos (CTM cultivadas e o concentrado de medula óssea) estão disponíveis comercialmente e são frequentemente utilizados na prática em equinos (Schnabel et al., 2013).

O principal objetivo da terapia celular é a regeneração de tecidos que geralmente não regeneram adequadamente, como músculo, tendões, ligamentos, meniscos e cartilagem. Dessa forma, medicina regenerativa procura

restabelecer a estrutura e funcionalidade de um tecido ou órgão lesado em sua completa normalidade ou próximo a esta, minimizando assim o risco de recidiva da lesão (Alves et al., 2011).

Atualmente 5400 testes clínicos com células tronco estão sendo realizados em todo o mundo, sendo 55% deles concentrados nos Estados Unidos da America (https://clinicaltrials.gov/ct2/results/map?term=stem+cells). Segundo Trounson et al. (2011) 24% desses estudos são relacionados a rejeições e doenças autoimune, e 23% com doenças degenerativas de ossos e cartilagens. Demonstrando a crescente progresso para o uso terapêutico desse tipo celular em diferentes enfermidades.

A aplicação intra lesional de CTMs pode melhorar a cicatrização por modular a inflamação aguda e minimizar a formação de tecido fibroso (Schnabel et al. 2013; Gutierrez-Nibeyro, 2011). O início precoce do tratamento com células-tronco é mais vantajoso do que quando já há formação de tecido cicatricial ou fibrose. Atualmente este objetivo pode ser alcançado quando utilizamos o concentrado de medula óssea, contudo este é constituído por uma população heterogênea de células, oferecendo uma quantidade muito reduzida de CTMs (Schnabel et al. 2013). Apesar do número de células necessário para atingir um nível terapêutico ainda ser desconhecido, a maioria dos estudos realizados *in vivo* com tratamento de tendinites tem mostrado resultados promissores com injeções intra lesionais contendo entre 5 a 10 milhões de CTMs (Gutierrez-Nibeyro, 2011; Carvalho et al. 2012).

Carvalho et al. (2012), fizeram o uso de CTMs derivadas do tecido adiposo associado ao plasma rico em plaquetas para a terapia da tendinite após 14 dias da indução da lesão, e observaram diminuição da inflamação e prevenção quanto à progressão da lesão tendínea nos animais tratados. Possivelmente este resultado foi devido à ação imunomodulatória apresentadas pela associação terapêutica. Posteriormente, Carvalho et al. (2013b) também relataram que lesões tendíneas tratadas com AdCTMs apresentaram melhora histopatológica quanto a organização e uniformidade da reparação tecidual quando comparado com o membro controle, incluindo baixa celularidade, redução do infiltrado inflamatório e baixa densidade fibroblástica, aumento do arranjo paralelo das fibras, do depósito de matriz extracelular e na expressão de colágeno tipo I.

Tipicamente as CTMs são obtidas do próprio paciente (autóloga), porém, o uso de CTMs provenientes de um cavalo doador (alogênica) e submetidas ao cultivo e a criopreservação cria a oportunidade de iniciar o tratamento logo após o diagnóstico e oferece uma população celular maior e mais homogênea em menor tempo.

No entanto, o transplante de células ou tecidos de um indivíduo para outro não geneticamente idêntico pode levar à rejeição do transplante devido a uma resposta imunológica. De forma geral, a rejeição ocorre entre 7 e 14 dias após o primeiro transplante e mais rapidamente depois do segundo transplante do mesmo doador, indicando que o receptor desenvolve uma memória contra às células transplantadas através da ativação da imunidade celular e humoral (Abbas et al., 2012).

Uma rede de células pertencentes ao sistema imune é responsável por mediar múltiplos processos que incluem o reconhecimento de antígenos próprios ou não próprios do organismo, sendo dessa maneira responsável por neutralizar toxinas, e eliminar patógenos e células tumorais. Há essencialmente dois componentes da resposta imune, a inata (não específica) composta por neutrófilos, mastócitos e macrófagos; e a adquirida. Os linfócitos são a base do sistema de defesa imune adquirida sendo divididos em linfócitos B e T. Os linfócitos T são responsáveis pelas reações imunes celulares, além de serem responsáveis pela ativação de outros tipos de células, como por exemplo os linfócitos B. Mais especificamente, os linfócitos T *helpers* reconhecem antígenos associados a molécula de MHC- II, regulando a produção e secreção de imunoglobulinas pelos linfócitos B (Galley e Webster, 1996; Arai et al., 1990).

A resposta imune e a inflamação são reguladas pela comunicação entre células através de substâncias solúveis denominadas de citocinas, que incluem quimiocinas, interleucinas (IL), fatores de crescimento e interferons (Galley e Webster, 1996).

A IL-1 é uma citocina mediadora da inflamação e da resposta a lesões. Apesar de incialmente se acreditar que a IL-1 era uma proteína produzida por macrófagos ativados, atualmente sabe-se que ela é secretada por diversos tipos celulares, entre eles fibroblastos, queratinócitos e linfócitos T e B. Essa citocina possui diversos efeitos biológicos, sendo um pirógeno endógeno responsável pela indução da febre. Além disso, atua como um fator ativador de hepatócitos,

estimulando a produção de proteínas de fase aguda pelo fígado e como um importante ativador de linfócitos T. Em adição a isso, a IL-1 pode interagir com outras citocinas como a IL-4 e ativar células B, aumentando a secreção de imunoglobulinas. A IL-1 é a principal molécula em reações inflamatórias, induzindo a produção de prostaglandina E2, colágeno e fosfolipase A2 (Arai et al., 1990).

O fator de necrose tumoral alfa (TNF-α) é produzido principalmente por macrófagos ativados, sendo considerado um importante mediador pro inflamatório e indutor da apoptose celular (WAJANT; PFIZENMAIER; SCHEURICH, 2003). Além disso, faz parte de uma rede complexa de citocinas, podendo ter uma ação sinérgica ou inibitória por controlar a síntese e expressão de outras citocinas como por exemplo a IL-1 (Collins e Grounds, 2001). Mioblastos expressam constantemente TNF-α, sendo que em lesões musculares os níveis de TNF-α aumentam drasticamente devido ao aumento de sua expressão pelas fibras musculares e pela infiltração de macrófagos. Sendo que a produção de TNF-α por fibras musculares está diretamente correlacionado com a regeneração tecidual e o tecido muscular regenera bem em presença de TNF-a (CHEN; JIN; LI, 2007).

Há evidencias de que as CT possuem capacidade de imunomodulação, interagindo com os linfócitos T de diversas maneiras. CTMs secretam mediadores solúveis como PGE2, fator de crescimento hepático, fator de crescimento transformador beta (TGF-β), interferon gama (IFN-γ), IL-10, fator inibidor de leucemia (LIF), antígeno leucocitário humano G (HLA-G), e indoleamine 2,3-dioxygenase (IDO), que agem diretamente nas células T e/ ou nos macrófagos modulando a sua atividade, principalmente inibindo a proliferação leucocitária. Além disto, as metaloproteinases derivadas da matrix de CTMs também tem o potencial de clivar receptores para IL- 2 (CD25) da superfície de linfócitos T reativos, resultando na redução da produção de IL- 2 e consequentemente reduzindo a proliferação de linfócitos (Peroni & Borjesson, 2011; Abbas et al., 2012). Sendo assim, as CTMs podem suprimir a proliferação de células T, induzia apoptose de células T CD8+, suprimir a ativação de células B e Th17, inibir diferenciação e maturação de células dendríticas e macrófagos e diminuir a produção de citocinas inflamatórias (Nauta & Fibbe, 2007; Cao et al., 2015).

Além disso, acredita-se que as CTM alogênicas equinas não induzem uma resposta imune complexa, uma vez que, similar a humanos e murinos, expressam em sua superfície celular MHC tipo I, mas não MHC tipo II. Sendo que, as moléculas MHC tipo II, tipicamente encontradas na superfície de células especializadas como macrófagos, células dendriticas e células B, estimulam células T a se diferenciar em citotóxicas ou “T Helper”, as quais produzem citocinas inflamatórias que destroem as células transplantadas (Borjesson & Peroni, 2011, Gutierrez-Nibeyro, 2011, Abbas et al., 2012).

Contudo, Schnabel et al. (2014), relataram que os cultivos de CTMs equinas provenientes da medula óssea (MoCTMs) apresentaram características heterogêneas quanto a expressão de MHC II, sendo que diferentemente de estudos realizados em humanos onde a expressão de MHC II somente foi observada nas primeiras passagens dos cultivos celulares (P1, P2), alguns animais foram fortemente positivos para MHC II até a oitava passagem celular (P8). Desta forma, concluíram que estes resultados podem estar relacionados a uma gama de fatores tais como genética, qualidade do aspirado medular, perfil imunológico e condições de cultivo, sendo que as MoCTMs podem ser mais propensas a esta heterogeneidade.

Em contradição a isto, Barberini et al. (2014) compararam a caracterização imunofenotípica e o potencial de diferenciação entre CTMs de origem de medula óssea, tecido adiposo e cordão umbilical de equinos, obtendo uma expressão mínima de MHC II em todos os grupos sem diferença estatística significante entre eles. Concluindo, portanto, que estas fontes de CTMs poderiam ser utilizadas com segurança em experimentos clínicos em equinos.

Mais recentemente, Pezzanite et al. (2015) demostraram que MoCTM alogênicas aplicadas pela via intradérmica podem desencadear uma forte e duradoura produção de anticorpos em equinos, entre 7 e 14 dias após a aplicação; concluindo que as CTM podem não ser imunoprivilegiadas como se acreditava originalmente, e que a aplicação alogênica desse tipo celular deve ser realizada com critério. Além disso discutiram que, apesar de não terem observado alterações sistêmicas significativas nos animais, a presença de anticorpos circulantes pode levar a destruição das CTMs alogênicas antes que estas tenham a chance de exercer seu efeito regenerador, sendo então prejudicial a terapia celular. Enfim, concluem que uma segunda aplicação pode

causar uma reação sistêmica significativa, sendo necessários mais estudos sobre o efeito imunogênico das CTMs alogênicas *in vivo*.

Carrade et al. (2011b) demostraram que a aplicação de CTMs alogênicas de origem placentárias na articulação de equinos não causou nenhum tipo de resposta adversa ou diferente daquela observada na aplicação de células tronco autólogas no mesmo tipo de tecido. Além disto, Carrade et al. 2011a, demostraram que a aplicação intra dérmica de células tronco alogênicas originadas no cordão umbilical não estimulou qualquer tipo de rejeição ou resposta de hipersensibilidade, mesmo quando os animais foram submetidos a aplicações repetidas. Estes resultados sugerem que as células tronco alogênicas do cordão umbilical poderiam ser administradas pelo menos duas vezes sem causar uma resposta imune.

Diversos experimentos clínicos com células alogênicas em animais e no homem estão sendo realizados baseados na premissa de que estas células são caracterizadas como sendo tolerogênicas. No entanto, como citado anteriormente, há evidências que indicam que estas células podem provocar alguma reação imune local, principalmente quando implantadas em tecido inflamado (BUJA & VELA, 2010).

Desta forma, a aplicação de CTMs alogênicas em um tecido hospedeiro íntegro possibilitaria a avaliação da reação inflamatória local após o implante destas células sem que haja interação de outros fatores, demostrando que as possíveis alterações foram realmente geradas pelo transplante celular (GALA et al, 2013).

O tecido muscular é ideal para a análise da relação topográfica entre os componentes do tecido em si, como fibras musculares, vasos sanguíneos e tecido conjuntivo, e células inflamatórias tais como linfócitos T e B, e macrófagos. Além disso, as fibras musculares são um grande alvo das reações imunológicas uma vez que suas células estão entre as maiores do organismo. Estas características possibilitam, uma análise precisa da relação entre as áreas de tecido muscular expressando marcadores de superfície imunologicamente relevantes e as células inflamatórias (HOHLFELD & ENGEL, 1992).

Estudos em camundongos com distrofia muscular relataram que o implante de CTMs influencia diretamente o mecanismo de inflamação no músculo distrófico. Foi observado que os animais tratados apresentaram

aumento da vascularização, redução de tecido fibroso, diminuição da inflamação e preservação da massa muscular. Além disso, observou-se uma redução de citocinas pró-inflamatórias (TNF e IL-6), espécies reativas de oxigênio, aumento de citocinas antiinflamatórias (IL-4 e IL-10) e macrófagos (PINHEIRO, 2011).

No estudo descrito por Zhang et al. (2007), demonstrou-se que CTMs coletadas da medula óssea de ratos regularam a resposta imune, reduziram a produção de mediadores inflamatórios (IL-1, TNF- α e TNF- β 1) e preservaram enxertos no estágio inicial de transplante.

Guest et al. (2008), induziram lesões no tendão flexor superficial digital de equinos e utilizaram transplantes alogênico e autólogo de células-tronco marcadas, coletadas de medula óssea como tratamento terapêutico. Os resultados foram analisados com 10 e 34 dias, observando a presença da maior parte das células marcadas com plasmídeo pCAG-EGFP-1 no local da lesão e de leucócitos tanto aos 10 dias quanto aos 34 dias em ambos os tipos de transplantes. Não foram observadas diferenças qualitativas entre os transplantes autólogo e alogênico com relação ao número de células marcadas e de leucócitos. Com relação à resposta inflamatória, o estudo demonstrou que tanto as células-tronco autólogas como alogênicas podem ser utilizadas sem estimular uma resposta imune mediada por células indesejável ao hospedeiro.

Salientando os poucos trabalhos descritos na literatura com o implante alogênico de CTMs principalmente na medicina equina, e a falta de concenso entre eles, o estudo do comportamento local das CTMs alogênicas, em comparação com a utilização do implante autólogo, possibilita diversas abordagens terapêuticas e laboratoriais.

Desta forma, o presente estudo objetivou avaliar os efeitos do implante de células tronco mesenquimais alogênicas derivadas da medula óssea (MoCTMs) em tecido muscular equino hígido a fim de qualificar e quantificar uma possível reação inflamatória local por marcadores inflamatórios (IL-1 β e TNF- α), parâmetros clínicos e ultrassonográficos., Sendo que, segundo nossa hipótese, essa reação seria mínima ou nula, permitindo assim o uso terapêutico de CTMs alogênicas em equinos.

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CAPITULO 2

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A clinical study of the inflammatory response after implantation of allogeneic mesenchymal stem cells in healthy horses

Background: The use of mesenchymal stem cells (MSCs) from a donor horse (allogeneic) creates the opportunity to initiate stem cell treatment immediately after a diagnosis. However, evidence suggests that these cells can cause local inflammatory reactions. This study aimed to evaluate the effects of transplanting allogeneic mesenchymal stem cells derived from bone marrow (MoCTMs) to validate the use of this approach in the clinical.

Methods: Three healthy horses were selected as donors and subjected to bone marrow aspiration for MSC cultures. Recipient animals were divided into six groups of 5 animals that were designated as follows: MSC 24h, implantation of allogenic MSC biopsy after 24 hours; MSC 7D, implantation of allogeneic MSCs biopsy after 7 days; MSC 14D, animals subjected to two applications of allogeneic MSCs with a 7-day interval and biopsy 7 days after the second application; PBS 24h, application of PBS and biopsy after 24 hours; PBS 7D, application of PBS and biopsy after 7 days; and PBS 14D, animals subjected to two applications of CTM PBS with a 7-day interval and biopsy 7 days after the second application. We aimed to study inflammation that could result from the allogeneic MSC

implantation in muscles of healthy horses by physical examination, hematology, ultrasound, thermography, histopathology and IL-1 β and TNF-α gene expression.

Results: Physical parameters of all of the animals remained within the normal range throughout the experimental phase. We observed that the second application of allogeneic MSCs from the same donor led to a significant increase in TNF-α gene expression.

Conclusion: Intra-muscular application of allogeneic MSCs is a clinically safe process, but it must be performed with discretion, especially after multiple applications. Additionally, we emphasize the importance of making constant progress in studies related to the immunogenicity of MSC to enable the safe clinical application of these cells.

Key words: mesenchymal stem cell, equine, rejection, muscle, inflammation

INTRODUCTION

Stem cells (SC) can be defined as cells with a great capacity for proliferation and self-renewal and can give rise to different cell lines [1]. Mesenchymal stem cells (MSCs) are typically obtained from a patient (i.e., autologous); however, the use of MSCs from a donor (allogeneic) that are subjected to culture and cryopreservation creates the opportunity to initiate treatment immediately after a diagnosis and offers a larger and more homogeneous cell population. However, transplantation of cells or tissues from one individual to another who is not genetically identical can lead to rejection because of an immune response [3].

Several clinical trials that used allogeneic stem cells in animals and humans have been carried out based on the premise that these cells are immunogenic [6, 7, 8, 9]. However, evidence exists that these cells can cause an immune reaction, especially when administered in inflammatory conditions [10].

Thus, the application of allogeneic MSCs in healthy host tissue allows for the evaluation of local inflammatory reactions following the implantation of these cells without interactions resulting from other factors [11].

Muscle is an ideal tissue for topographic analyses of the relationship between components of the tissue, including muscle fibers, blood vessels, connective tissue, and cells [12].

This study aimed to evaluate the effects of the implantation of allogeneic mesenchymal stem cells derived from equine bone marrow in healthy muscle tissue to qualitatively and quantitatively characterize a possible local inflammatory reaction, based on measurements of inflammatory markers (IL-1β and TNF-α) along with clinical, histopathological and sonographic parameters.

Given that, we expect the reaction to be minimal or absent, enabling therapeutic use of allogeneic MSCs in horses.

MATERIALS AND METHODS

Experimental design

The experiment was conducted in the Department of Veterinary Surgery and Anesthesiology of the School of Veterinary Medicine and Animal Science-UNESP, Botucatu and approved by the Ethics Committee (CEUA) with the protocol number 186 / 2013- CEUA.

A total of 3 horses were selected as donors, and 5 horses (different from the donors) per group were selected as recipients. All animals were healthy, aged between 5 and 10 years old, weighed between 300 and 400 kg and were of a mixed breed.

Recipient animals were submitted to intra-muscular-implantation of allogenic MSCs or PBS application (control) and were divided into the following six groups:

- **MSC 24h-** implantation of allogenic MSC and biopsy after 24 hours;
- **MSC 7D-** implantation of allogeneic MSCs and biopsy after 7 days;
- **MSC 14D-** two implantations of allogeneic MSCs with a 7-day interval between then and biopsy 7 days after the second implantation;
- **PBS 24h-** application of PBS and biopsy after 24 hours (MSC 24h control);
- **PBS 7D-** application of PBS in the and biopsy after 7 days (MSC 7D control);

- **PBS 14D-** two applications of PBS with 7-day interval between then and biopsy 7 days after the second application (MSC 14D control);

The moments of application, avaliation and biopsy are represented in Figure1.

Bone Marrow Aspiration

Three healthy horses were used as donors. The animals were selected after physical examination and ultrasound of the sternal region.

After a physical examination and ultrasound of the sternal region, animals were sedated with 10% Xylazine (Sedazine, Fort Dodge, Campinas- SP, Brazil) at a dose of 0.5 mg/kg intravenously. Then, the sternum region was shaved (5 x 20 cm), and the site of aspiration was identified under ultrasound guidance. A local anesthetic block was induced with 15 ml 2% lidocaine without vasoconstrictor in subcutaneous tissue, followed by scrubbing with 0.5% chlorhexidine.

Bone marrow aspiration was performed using an 8 G, 15 cm long Komiyashiki needle to obtain a final volume of 20 ml bone marrow per animal. This material was then taken to the laboratory to separate the mononuclear fraction and subsequently establish mesenchymal stem cell cultures.

Isolation and culture of MSCs

Samples were deposited on Ficoll-Paque Plus® (p = 1.077) (Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 1:1 and centrifuged at 251 G for 30 minutes. After centrifugation, the mononuclear fraction was separated, and the resulting cell pellet was washed once with PBS to remove the

Ficoll-Paque Plus ®.

After washing, the pellet was resuspended in 1 mL DMEM (Dulbecco's Modified Esadle's Medium, Gibco, Grand Island, NY, USA) and seeded in 75 cm^2 cell culture flasks using DMEM Knockout® culture medium, 10% fetal bovine serum (FBS) containing penicillin, streptomycin and amphotericin B. Culture vials were taken to the incubators at 37°C and 5% CO_2.

The culture medium was changed every three days, and when cultures reached a confluence of at least 70% of the plate, cells were suspended based on the technique described previously in horses [13]. Cells were cultured to the third passage (P3) and then prepared for cryopreservation.

The cell count was performed using an ordinary microscope in a Neubauer chamber, and the cell viability was evaluated based on the exclusion of trypan blue (Gibco® Invitrogen Corporation).

For such, 100 µL of the sample were diluted in 100 µL trypan blue and counting of viable cells was carried out based on the following mathematical calculation:

$$\text{Cell n°/ mL} = \frac{\text{nº viable cells counted}}{4} \times 2 \times 10^4$$

For freezing, the cell suspension was centrifuged at 251 G for 10 minutes, and samples were then divided into cryotubes at a concentration of 1×10^6 cells per mL of a Bambanker ® (Lymphotec Inc., Tokyo, Japan) cryopreservation medium and stored in a –80°C freezer.

Implantation of MSCs

Recipient animals were selected after physical and hematological examinations and ultrasound of the superficial gluteal muscle area was carried out, excluding any systemic or local changes.

Previously cryopreserved cell samples were thawed one week before the applications in a water bath at 37°C for 2 minutes and put into culture until 10×10^6 cells per animal were expanded.

After reaching cell confluence of at least 80%, cells were resuspended, and the concentration and viability were determined according to a previously described method. This process yielded a total of approximately 10×10^6 cells with 95% viability at the time of application.

The applications were guided by ultrasound to determine the exact location and depth and were held at a single point in the superficial gluteal muscle using a hypodermic needle of 30×07 mm in a total volume of 3 ml PBS with a depth of approximately 1.0 cm.

The local inflammatory response was evaluated after cell implantation by physical, thermography, and ultrasound examinations.

Physical exam

Heart and respiratory rates, body temperature, and examinations of the application site were made at the evaluations, according to the experimental design (Figure 1).

The local physical examination consisted of an assessment of the presence of pain, warmth, and swelling in the application area based on local palpation by three examiners and was graded as absent (0), mild (1), moderate (2) or intense (3). The presence of pain was analyzed by putting pressure on the application site and evaluating the response of each animal. Warmth and swelling were analyzed in a subjective manner according to the opinion of each examiner.

Thermographic examination

The day before the first thermographic exam, the animals were submitted to trichotomy of the gluteus medius region and kept in stall without exposure to the sun, until the end of the experiment. Analyses were performed indoors, without any manipulation of the area.

Examinations were performed with a FLIR Systems FLIR SC660 model thermographic camera. The images and values of temperatures were obtained using FLIR Systems QuickReport Software.

We then designed a rectangle, including the area adjacent to the application site, and based on the maximum and minimum recorded temperature we calculated the local mean, as shown in Figure 2. This procedure was performed, according to the experimental design, on all animals in each group. The evaluation times were before, during and 24 hours after application, and every 48 hours thereafter until the time of biopsy, according to the experimental design (Figure 1).

Standardization of temperatures was performed based on the room temperature. Thus, we used two mathematical equations in which the first was

the ratio between the room temperature of each subsequent day (T_{amb_i}) and room temperature on the first day of evaluation (T_{amb_0}), resulting in the value θi (theta), as shown below.

$$\emptyset_i = \frac{T_{amb_i}}{T_{amb_0}}$$

The second was the standardization of the average temperature of the subsequent days, divided by the calculated value of θi (theta), as demonstrated in the following equation. This procedure was based on the international methodology of standardization for atmospheric temperature [11].

$$T_{lesão_{i_{corr}}} = \frac{T_{lesão_i}}{\emptyset_i}$$

Repeated measures analysis were performed for comparisons of the temperature at the evaluation moments ($P < 0.05$). A *t*-test (Student) was used to compare this parameter between groups based on a 5% significance level. When the analysis was found to be significant, the Tukey test was performed to compare means ($P < 0.05$).

Ultrasonographic examination

Ultrasonographic examinations were performed with ultrasound equipment My Lab 70 (The Esaote Group, Italy), with linear transducer, frequency of 18 MHz, 95-100% gain, depth of 3 cm and 0.5- 1, 5 cm focus. The evaluation was performed in the longitudinal plane, evaluating change in

echogenicity, echotexture and parallelism between the fibers and characterized for the presence of hypoechoic areas, with a loss of parallelism between fibers (HYPO); hyperechoic linear imaging between fibers (HYPERLI); hyperechoic region/area (HYPER); and formation of hyperechoic punctiform images (HYPERPI). The normal parameter standardized before the application (M0) and characterized by the presence of hypoechoic myofibrils separated by thin parallel hyperechoic septa.

The evaluation time points were before, during and 24 h after application, and every 48 h thereafter until the time of biopsy, according to the experimental design (Figure1).

Statistical analyses were performed using the chi-square test comparing the proportion of animals with each sonographic change, between groups and moments, with a significance level of 5% ($P < 0.05$).

Muscle biopsies

Muscle biopsies were performed following the experimental design (Figure1) 24 hours after application (MSC 24h and PBS 24h), 7 days after application (MSC 7D and PBS 7D) and 7 days after subsequent applications with 7-day interval between them (MSC 14D and PBS 14D).

For this, the animals were sedated with xylazine 10% (Sedazine, Fort Dodge, Campinas SP, Brazil) at a dose of 0.5 mg/kg intravenously. Then, the local anesthetic blockage was maintained with 2% lidocaine without a vasoconstrictor in the subcutaneous tissue, skirted the area to be biopsied, and followed by scrubbing with chlorhexidine 0.5%.

The skin incision was performed with a number 24 scalpel blade, allowing access to the superficial gluteal muscle. Once the muscles were isolated, a fragment of approximately 2 cm^3 was taken from the area previously marked at the time of application. The muscle fragment was separated, divided and properly stored in 10% formaldehyde for histopathological examination or liquid nitrogen for gene expression analyses. The suture of the surgical wound was performed in two planes by the approach of the subcutaneous tissue and skin.

Histopathological Exames

Transverse histological sections of paraffin blocks were made. Sections were stained with hematoxylin & eosin (H&E) and examined under an optical microscope with blind assessments for the experimental group. For this evaluation, in addition to the samples of the experimental groups, there were 5 samples of otherwise healthy muscle tissue (without application) and 5 samples of muscle tissue subjected to introduction of a 30×07 mm needle without the administration of cells or PBS.

Morphological analyses were performed according to Brasileiro et al. [13] with a magnification of 100 and 400 times to evaluate the presence of neutrophilic infiltrates, edema and degeneration/necrosis of the muscle fibers; being graded as absent, mild, moderate or severe as described in Table 1.

Values were obtained by calculating the arithmetic mean of the number of cells/fiber viewed in 10 fields counted per slide, according to the methodology described by Brasileiro et al. (2007).

Images were captured by a microscope connected to a camera and and analyzed using Leica Qwin 3.0 images analysis program.

Table 1. Graduation and criteria used to evaluate the parameters of histological examinations of the superficial gluteal muscle of horses by hematoxylin and eosin staining.

	Score			
	0	**1**	**2**	**3**
	Absent	**Mild**	**Moderate**	**Severe**
Edema	Absent	1 spacing between fibers per field (100X)	2-4 spacing between fibers per field (100X)	>5 spacing between fibers per field (100X)
Neutrophilic infiltrate	Absent	1-10 neutrophils per field (400X)	11-20 neutrophils per field (400X)	>20 neutrophils per field (400X)
Degeneration of the muscle fibers	Absent	1 degenerated fiber per field (100X)	2-3 degenerated fiber per field (100X)	>4 degenerated fiber per field (100X)

Real-time polymerase chain reaction (RT-qPCR)

Immediately after biopsy of muscle tissue, samples were separated and stored in cryotubes, which were immersed directly into liquid nitrogen for freezing. These were stored in a freezer at –80°C until extraction of RNA. Furthermore, 5 samples of healthy muscle tissue were biopsied and frozen to be used as a negative control.

To perform RT-qPCR, RNA was extracted using TRIzol ® reagent (Invitrogen ref. 15596-018) according to manufacturer's instructions. Then, RNA purification was performed using DNase I Amplification Grade (Invitrogen, ref. 18068-015) and conversion of mRNA to cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ref. 4368813), according to manufacturers' recommendations. The cDNA integrity was analyzed using Bioanalyzer equipment.

Primers were designed with Primer Express 3.0 software (Apllied Biosystems) for the target genes IL-1-β and TNF-α and endogenous genes glyceraldehyde - 3- phosphate dehydrogenase (GAPDH), glucuronidase beta (GUSB), actin beta (ACTB) and titin (TTN). The specificity of the primers of each gene was assessed by NCBI website BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/), to foward and reverse sequences.

RT-qPCR reactions for each primer were standardized, and we determined the efficiencies of reactions for each primer pair using standard curves from a control sample of cDNA (sample with a good quality RNA).

Amplification reactions were performed in 384-well plates, containing triplicates of each sample. In each well, 10.0 μL of a solution composed of 8.5 μL of the reagent SYBR Green PCR Master Mix was added (Applied Biosystems) and primers (sequence forward and reverse) and 1.5 μM 10 μL of cDNA (dilution 1:10). The reaction was performed in QuantStudio 12K Flex equipment - (Applied Biosystems).

Amplification reaction for all primer consisted of 40 cycles of 15 seconds at 94° C and 1 minute at 60 ° C. Dissociation curves were included at the end of

each run to determine the specificity of the PCR products visualized by a single peak of the amplified product.

The analysis of each reaction was performed by determining the baseline, which consisted of the quality of the fluorescence read during the initial cycles of PCR. Subsequently, we determined the threshold, which is selected in the exponential phase of the PCR curve. The point at which the intersection with the threshold amplification curve occurs is determined as threshold cycle (Ct). All samples were compared at the same threshold, but Ct varies with the cycle at which the amplification curve crosses the threshold. Therefore, each sample has a specific Ct value which is related to the amount of cDNA of the gene in question in the sample. Relative quantification (RQ) was calculated using the two-ΔΔCT method (Ct: threshold cycle; ΔCt – Ct of the target gene minus the Ct of the endogenous gene (geometric mean of the Cts of the endogenous genes); ΔΔCt – ΔCt muscle sample minus the ΔCt the normal sample). Genes that have RQ ≥2 will be considered to be positively regulated genes, and genes that have RQ ≤ 0.5 will be considered negatively regulated genes.

RESULTS

Physical exam

All physical parameters such as heart rate, respiratory rate and body temperature remained within the normal range for all animals throughout the experimental phase.

Data about the presence of heat, pain and swelling on the application site are shown in Figures 3–5.

At 24 h post-application (M24h), 20% of animals in the MSC24h group exhibited a mild increase in local temperature, and 20% had a moderate increase; In contrast, only 40% of animals in the PBS24h group showed a mild increase in temperature.

All of the animals in the MSC7D group (100%) presented an increase in temperature (40% mild and 60% moderate), while at day seven after application (M7 days) only 20% of these animals still showed a mild increase in local temperature; the other animals had returned to normal. In the PBS7D group 60% of animals had local temperature increase between moderate (20%) to mild (40%) at M24h, but at M7days, all animals had returned to normal. Most animals in group MSC14D and PBS14D showed mild local temperature increase at M24h (80% for MSC14D and 60% for PBS14D), while by 7 days after the second application (M14 days) all animals had returned to normal.

At 24 h post-application (M24h), 20% of the animals in the MSC24h group exhibited mild swelling at the site of application, while no animal from PBS24h showed this change. In MSC7D group, 60% of the animals had mild swelling 72 hours after application (M72h), and in the M7days, 20% of animals showed moderate swelling. In PBS7D group, 20% of animals showed moderate swelling at M72h and 7 days after application (M7dias). Most animals in the MSC14D group showed mild sweling at M72h (60%) and 7 days after the second application (M14 days) all animals had returned to normal. Only 20% of animals in the PBS14D group had mild swelling at M72h.

At M24h, 20% of the animals in MSC24h group and 60% of the animals in PBS24h group presented pain upon palpation of the application site. Animals in the MSC7D group had mild (40%) to moderate (20%) pain at M24h and M96h,

while at M7 days only one animal had severe pain. In the MSC14D group, 40% of the animals showed mild pain 72 hours after the first application, while 24 hours after the second application (24 M2) 80% of animals showed mild pain; however, by M14D no animals showed pain.

Thermographic examination

Measurements of local temperature by thermography are shown in Tables 2–4.

Table 2: Mean and standard deviation of the local temperature (° C) measured by thermographic examination in MSC 24h and PBS 24h.

Group	Temperature (ºC)		P
	M0	M24h	
MSC 24h	31,48 ± 1,26	28,42 ± 2,28	**0,105**
PBS 24h	31,78 ± 1,13	28,72 ± 2,36	**0,054**
P	**0,713**	**0,844**	

Table 3: Mean and standard deviation of the local temperature (° C) measured by thermographic examination in G2 and G5.

Group	Temperature (°C)					P
	M0	M24h	M72h	M96h	M7D	
MSC 7D	**27,84 ± 1,37**	**27,53 ± 1,37**	**29,07 ± 1,77**	**27,99 ± 1,26**	**28,03 ± 1,63**	**0,395**
PBS 7D	**27,82 ± 1,02**	**26,86 ± 0,51**	**28,98 ± 1,62**	**27,78 ± 0,86**	**27,98 ± 1,47**	**0,143**
P	**0,980**	**0,091**	**0,937**	**0,758**	**0,962**	

Table 4: Mean and standard deviation of the local temperature (° C) measured by thermographic examination in G3 and G6.

Moments		**Groups**		**P**
		MSC 14D	**PBS 14D**	
1° application (M)	**0h**	36,06 ± 0,34 bc	36,10 ± 0,59 bcd	**0,898**
	24h	37,16 ± 0,46 b	36,86 ± 0,41 bc	**0,338**
	72h	37,15 ± 0,60 b	37,25 ± 0,59 b	**0,792**
	96h	38,84 ± 0,94 a	38,91 ± 0,87 a	**0,913**
2° application (M2)	**0 h**	34,12 ± 0,54 d	33,98 ± 0,52 ef	**0,685**
	24 h	34,35 ± 0,99 d	34,60 ± 0,62 def	**0,648**
	72 h	34,04 ± 1,12 d	33,61 ± 0,58 f	**0,466**
	96 h	35,33 ± 1,30 cd	35,29 ± 1,25 cde	**0,957**
	14 dias	32,38 ± 1,10 e	33,48 ± 1,40 f	**0,202**
P		**<0,001**	**<0,001**	

Means followed by the same letter in the column do not differ statistically by Tukey test (P> 0.05).

No significant difference was detected in comparisons of the MSC24h and PBS24h, and MSC7D and PBS7D groups at any of the time points (P> 0.05).

The MSC14D and PBS14D groups at the time 96h (M96h) had statistically higher temperature compared to the other time points (P <0.05). It was also noted that in the time points leading up to the second application (M0, M24h, M72h and M96h), the temperatures were significantly greater then the other time points, and 7 days after the second application (M14 days) the MSC14D group showed significantly lower temperature compared to all other time points.

There was no significant difference between the groups at any time point in this study.

Ultrasonographic examination

The ultrasonographic changes are shown in Figure 6.

Ultrasonographic examination revealed the a hyperechoic linear image (HYPERLI), suggestive of the presence of material between the fibers 24 h post-application in all experimental groups, with a significant difference between the groups that received stem cells and their respective controls (application PBS), with the exception of the MSC14D and PBS14D groups wich had equal values after the first application, and differed only after the second application. These images were not observed in subsequent evaluations.

Hematoma, which is characterized by regional hyperechoic image (HYPER) formation, was observed in all groups within the first 24 h after application and persisted until the time of biopsy. There was only a significant difference between the MSC14D and PBS14D 24h after the first and the second application and 96 hours after the second application, of which the MSC14D group included a higher percentage of animals with this change, reaching 100% in M2 24h and M2 96h.

Edema was assessed by the presence of hypoechoic areas with loss of parallelism between the fibers (HYPO). At 72 h, all groups had animals with HYPO areas. The number of animals in the MSC7D group was significantly greater than its control (PBS7D) group 24h after the application until the time of biópisa. Additionally in this group 96 h after application, 80% of animals exhibited this alteration, with a reduction of 20% at the time of biopsy. The PBS7D group remained constant with 40% of the animals showing this change between 72 and 96 h after application, with a reduction of 20% at the time of biopsy. The PBS14D

group showed HYPO areas 24 and 72 h after the first and second application, while the MSC14D group only at 24 and 72 h after the first application. All groups differed significantly during these times, with the exception of MSC14D and PBS14D groups at M72h.

We observed hyperechoic punctiform images (HYPERPI) between muscle fibers that were suggestive of fibrin deposition in MSC7D, MSC14D, PBS24h and PBS7D groups at all times, from 24 h after application until biopsies muscle. However, a significant difference was observed only between the MSC7D and PBS7D groups at M96h and M7days, and PBS7D was significantly greater. However, the MSC14D and PBS14D groups did not differ significantly and contained the greatest percentage of animals with this type of images, as at the time of biopsy 100% of the animals in both groups showed HYPERPI.

Histopathological Exames

Histopathological avaliations are represented in Figure 7.

Mild neutrophilic infiltrate was observed only in MSC14D group. All animals in all groups, except in the NC group, showed spacing between muscle fibers, indicationg the presence of mild edema. All groups, except the NC and MSC7D groups, showed severe fibre degeneration. The NC group showed mild muscle degeneration and moderate muscle degeneration in the MSC7D group.

Furthermore, in MSC7D and MSC14D groups, there was a obvious presence of fat and mononuclear cell clusters between the fibers when compared to the needle, NC, MSC24h, PBS24h, PBS7D and PBS14D groups.

There was no significant statistical difference between the groups.

In Figure 8 e 9, there is photomicrographs of the muscle biopsy in the NC and needle groups are shown. In Figure 10, a photomicrographs of muscle biopsies of MSC24h, MSC7D, MSC14D, PBS24h, PBS7D and PBS14D groups are shown.

Real-time polymerase chain reaction

Data showing the relative expression levels of IL-1β and TNF-α when compared to a negative control (healthy muscle) are shown in Figures 11, in which the relative expression of TNF-α differed from the negative control in all groups, except the PBS24h group.

When we compared the groups that received stem cells implantation (MSC24h, MSC7D and MSC14D), we observed that the MSC14D group showed higher relative expression of TNF-α, while the other groups did not significantly differ between each other (Figure 12).

When we compare IL-1β and TNF-α expression in groups that received stem cells implantation with their respective controls (PBS application), only TNF-α expression in group MSC14D was statistically higher than its respective control (PBS14D; Figure 13).

Discussion

In this present study, we evaluated the acute inflammatory response following the application of allogeneic mesenchymal stem cells derived from equine bone marrow in the gluteal muscle of higid horses. No animal showed clinically significant changes during the experimental phase, as the vital parameters remained within normal rates. By physical examinations by palpation of the application site, some animals showed pain, heat and swelling in the first 24 to 72 h after application, but the pain returned to normal by the time of biopsy, with no significant difference between the groups.

According to Vukmanovic-Stejica et al. [14] the cellular immune response is initiated a few hours after contact with an antigen, reaching a peak at 48 to 72 h post-application, followed by regression within 10 to 14 days. Our findings are consistent with this statement because the clinical changes found at the application site occurred between 24 and 72 h after application and resolved within 7 days.

Carrade et al. [15] obtained similar findings after the intradermal application of autologous and allogeneic mesenchymal stem cells derived from the umbilical cord in horses, and did not observe hematological changes in the animals, and observed more frequant local reactions, such as wheal formation, at 48 to 72 h after application, followed by resolution within 7 days. Moreover, Pizzanite et al. [16] performed the intradermal application of mesenchymal stem cells derived from bone marrow in horses, and observed wheals formation at the application site followed by resolution during the experimental phase, with no other physical or hematological changes.

By thermographic examination, the MSC14D and PBS14D groups had a higher local temperature at time 96 h (M96h), which was significantly different from the other time points ($P < 0.05$), but not between groups. Therefore, we can consider that the local temperature increase was a consequence of a local reaction to the application rather than the presence of cells.

Ultrasonography revealed the persistence of stem cells and PBS up to 24 h after the applications, and those groups submitted to stem cell implantation had a significantly higher percentage of animals with this change compared with the respective controls (application with PBS), suggesting that cells remained longer at the site of application then the vehicle used for the application (PBS). We also noted hyperechoic regions that were suggestive of hematoma. Animals in the MSC14D group had a higher percentage of this change 24 h after both applications and 96 h after the second application, which were significantly different from the PBS14D group at those time points. The formation of edema was observed between 24 and 72 h after application, and it was significantly higher in the MSC7D group when compared with its control. However, the MSC14D group showed no edema formation after the second application. This finding suggests that animals subjected to the application of stem cells showed a greater local reaction 7 days after application, but not after the second application of MSCs.

However, the presence of fibrin deposition evidenced by hyperechoic punctiform images was significantly higher in the PBS7D group when compared to the MSC7D group at M96h and M7 days, suggesting that the presence of cells might have inhibited fibrin deposition. Several studies reported a reduction in liver fibrosis and inflammatory responses after cell therapy mainly because of less

collagen production and the secretion of cytokines and growth factors, such as IL-6 receptor antagonist of IL-1, iNOS, and MMP9, which have anti-fibrotic and anti-apoptotic effects [17, 18, 19, 20].

Adult MSCs from various sources have been reported to be immunoregulatory, with great potential for the regulation of T lymphocytes by inhibiting their proliferation, survival or differentiation and via the release of cytokines, such as prostaglandin E2 (PGE_2), human leukocyte antigen G5 (HLA-G5), induced nitric oxide synthase (iNOS), transforming growth factor-beta (TGF-β), leukemia inhibitory factor (LIF), and interleukin 10 (IL-10) [10, 22, 23]. However, the MSC immune suppression ability is not consistent in all *in vivo* studies because some reports that the allogeneic application of stem cells can induce cellular and humoral immune responses [16], while others reports indicate that the cells do not cause rejection, even after multiple applications [7].

Based on the changes observed by histopathological examination, we found that insertion of the needle and the presence of fluid between the muscle fibers was sufficient to elicit a local response, even causing the degeneration of muscle fibers. Thus, no histopathological changes were attributed to the presence of cells, but rather to a mechanical insult to the muscle fibers generated by the needle and volume of liquid injected. However, the second application of MSCs may have led to increased recruitment of neutrophils to the site of application. Furthermore, there was a clear presence of fat and mononuclear cells between the fibers that agglomerated in groups MSC7D and MSC14D.

Gala et al. [11] applied CTM in the higid muscle of mice and demonstrated that injected cells were present in clusters at the site of implantation 24 h after application with a significantly greater presence of mononuclear inflammatory

infiltrates around the MSC when compared with the control. Vela et al. [24] also observed clusters of cells surrounded by lymphocytes and macrophages after the implantation of MSCs in myocardial tissues and dogs. Both studies did not report any other type of change in local inflammation.

Although our study was not carried out by labeling MSCs, macrophages or lymphocytes, based on previous studies, we can assume that the cell clusters in the images found in groups MSC7D and MSC14D are the cells that we injected, possibly surrounded by a mononuclear infiltrate. Regarding the presence of fat, we can propose the following two theories: the MSCs may be suffering spontaneous differentiation to fat tissue at the application site or the MSCs are causing degeneration of the muscle fibers. Further studies will be necessary to test these theories.

For the gene expression of IL-1β and TNF-α in the muscle tissue at the application site, the MSC14D group showed significantly greater expression of TNF-α, while the relative expression of IL-1β was not significantly different between groups. Gata et al. (2013) performed a study similar to ours by evaluating the gene expression levels of IL-1α, IL-1β, IL-6 and TNF-α 24 h after the application of allogeneic MSCs in the higid muscle of mice. However, in contradiction to our results, they reported an increase in the gene expression of IL-1α and IL-1β, but not TNF-α. They also reported that these findings were significantly higher when compared to healthy muscle or a group submitted to the application of PBS. However, the application of PBS was sufficient to cause an increase in the gene expression of IL-1 when compared with higid muscle.

The gene expression of IL-1 occurred within 30 minutes after exposure and may last for hours, rapidly inducing the expression of other genes by various

cell types, such as monocytes, macrophages and endothelial cells [25]. Thus, the expression of IL-1 triggers the expression of other cytokines, such as TNF-α, and vice versa. TNF-α has a subsequent activity that mainly induces cell apoptosis [26]. Therefore, we infer that a 24-h period may have been too long to detect significantly higher expression of IL-1β; however, the sequential application of MSCs caused increased expression of TNF-α. This factor may be related to a specific immune reaction after the sensitization of an animal by the first application as the production of TNF-α is related to the recruitment of macrophages [27].

There is *in vitro* evidence that when in contact with pro-inflammatory cytokines, MSCs can be induced to express surface markers, such as MHC II, leading to triggering of a cellular immune response [9]. Pizzanite et al. [16] reported strong and long-lasting antibody production after the application of allogeneic intradermal MSCs in horses, and concluded that although a single dose of allogeneic MSCs was sufficient to elucidate a clinical response, there is a possibility that multiple applications might lead to a strong immune response that is harmful to the patient.

Additionally, we consider that although there was no significant clinical change, there is a possibility that these cells are recognized as foreign and will be eliminated before they can exert their therapeutic role. Zang et al. [28] injected autologous and allogeneic MSCs into mice and evaluated their persistence by cytofluorometry analysis, noting that most autologous MSCs were detected throughout the experimental period (40 days), although the allogeneic MSCs died within 20 days. In addition, after the second application of MSC, these cells underwent rejection sooner up to 2 days after application. This is a significant

point that we will evaluate in future experiments, as it directly influences the therapeutic results.

Finally, we conclude that the application of allogeneic MSCs is a clinically safe process, but it must be carried out with discretion, especially after multiple applications. We also emphasize the importance of constant progress in studies related to the immunogenicity of MSC, so there is a better understanding of the actions, reactions and dynamics of these cells to enable their safe clinical application, not only in veterinary medicine but also in humans.

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Figures

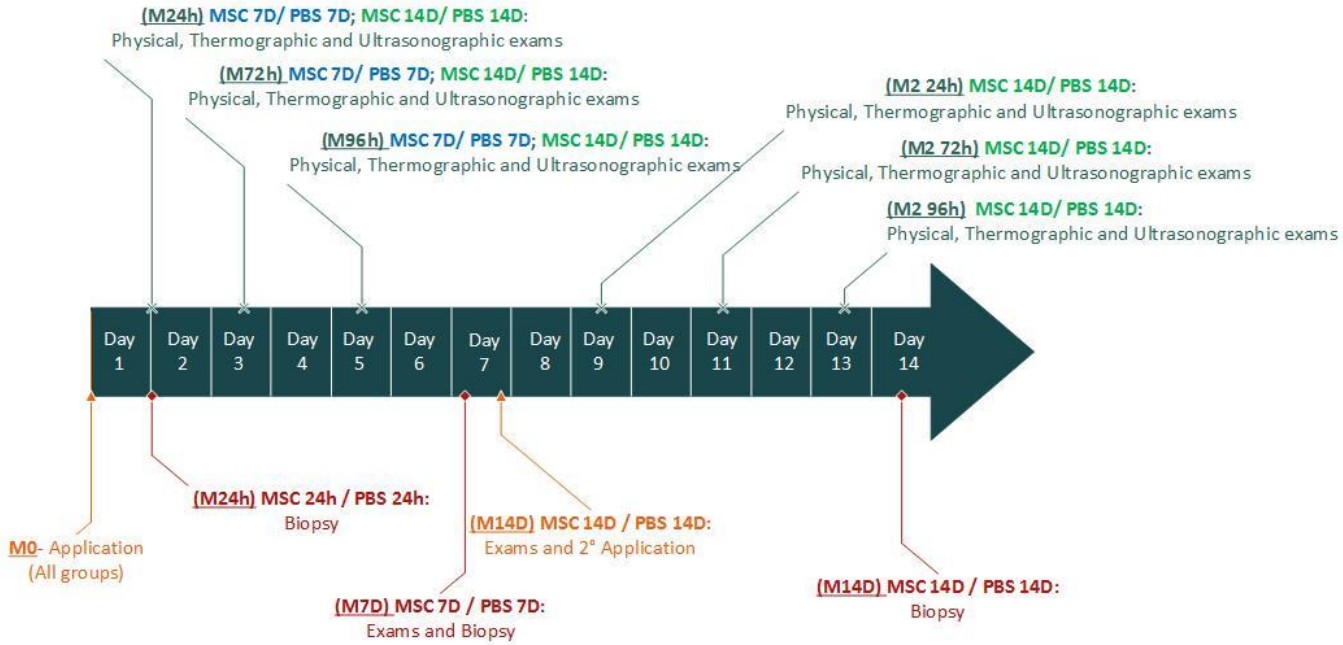

Figure1: Experimental design: moments of application, avaliation and biopsy.

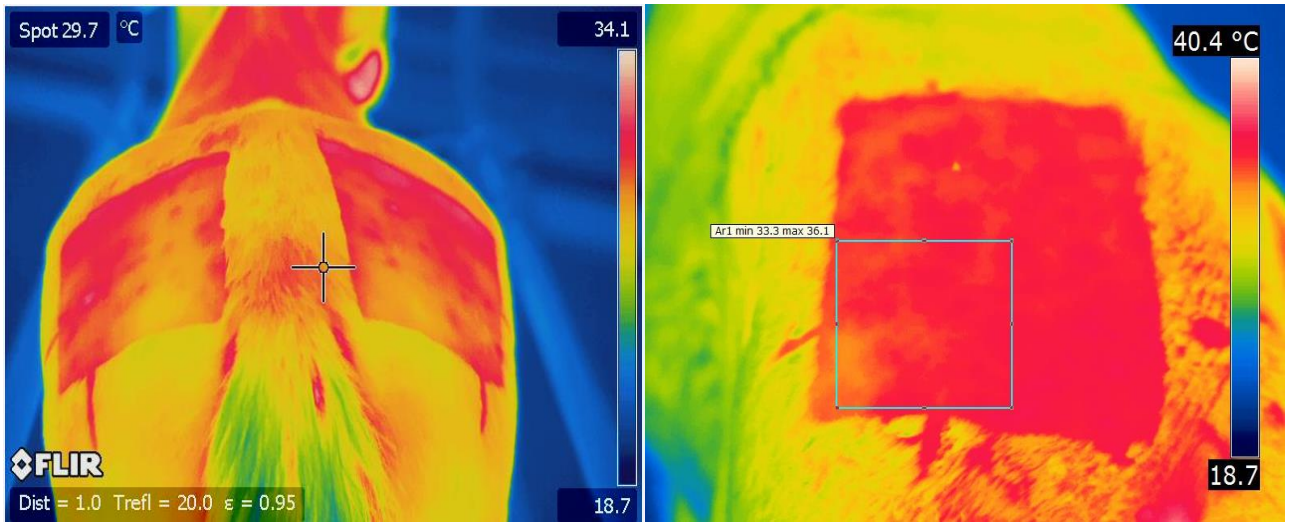

Figure 2: Measurement of local temperature by thermography. The projection of a rectangle including the adjacent area and de area of application was performed, being provided the maximum and minimum recorded temperature and calculated the mean temperature.

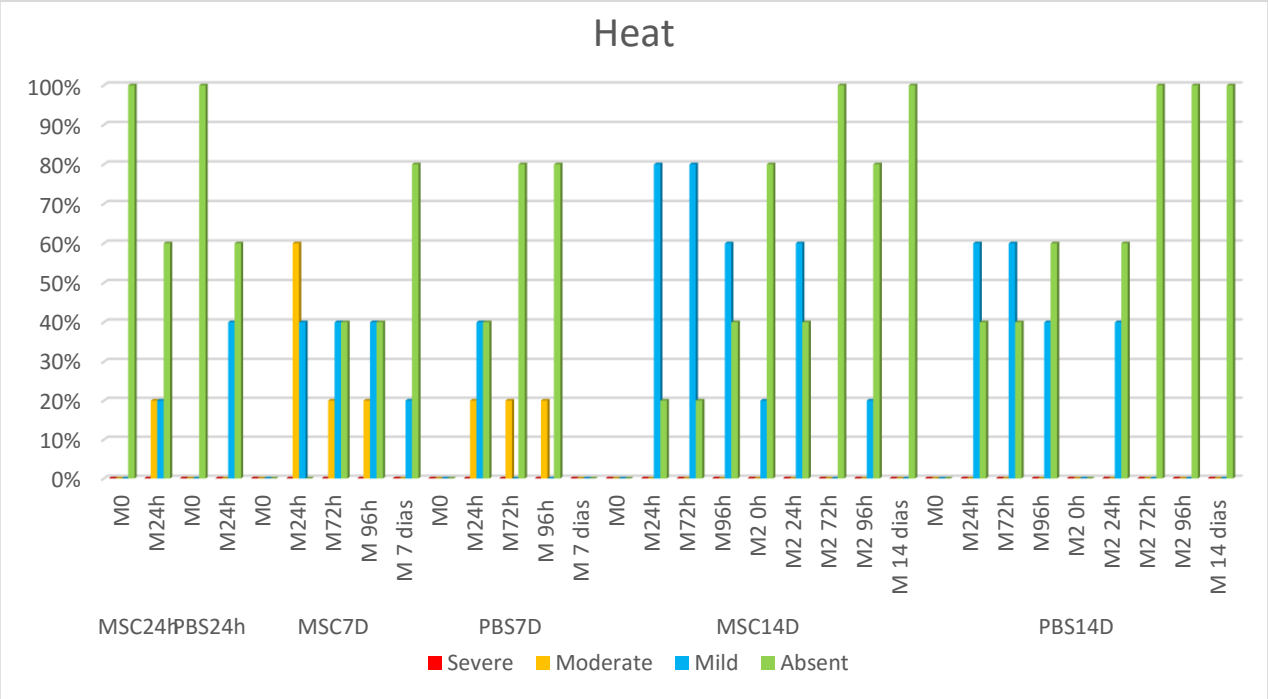

Figure 3: Percentage of animals with heat at the site of application: classified as severe, moderate, mild or absent throughout the experiment for all experimental groups.

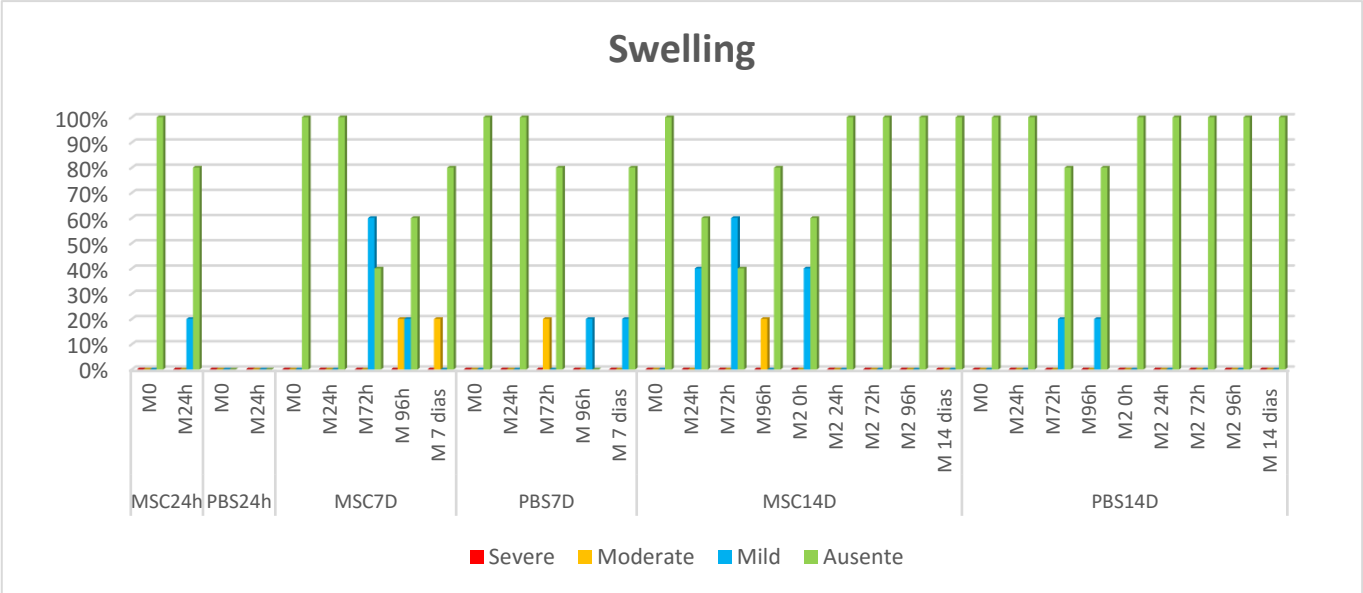

Figure 4: Percentage of animals with swelling at the site of application: classified as severe, moderate, mild and absent throughout the experiment for all experimental groups.

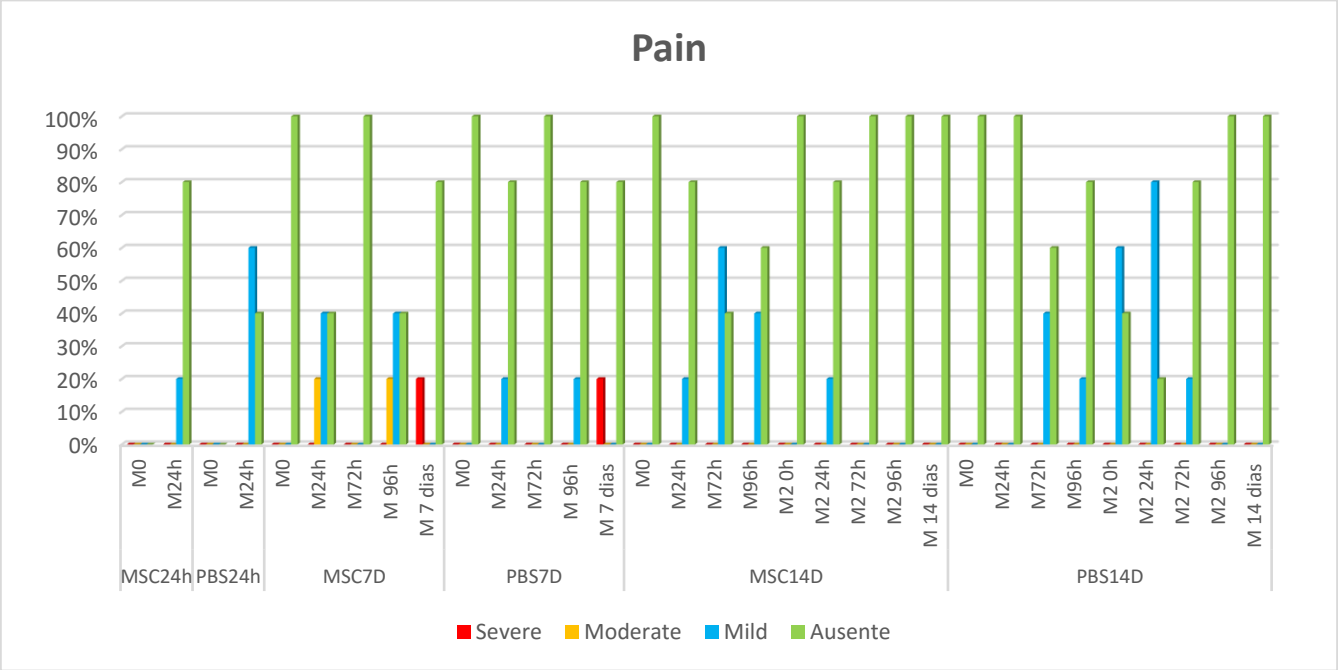

Figure 5: Percentage of animals presenting with pain at the site of application: Animals were classified as having intense, moderate, light or absent pain throughout the experiment for all experimental groups.

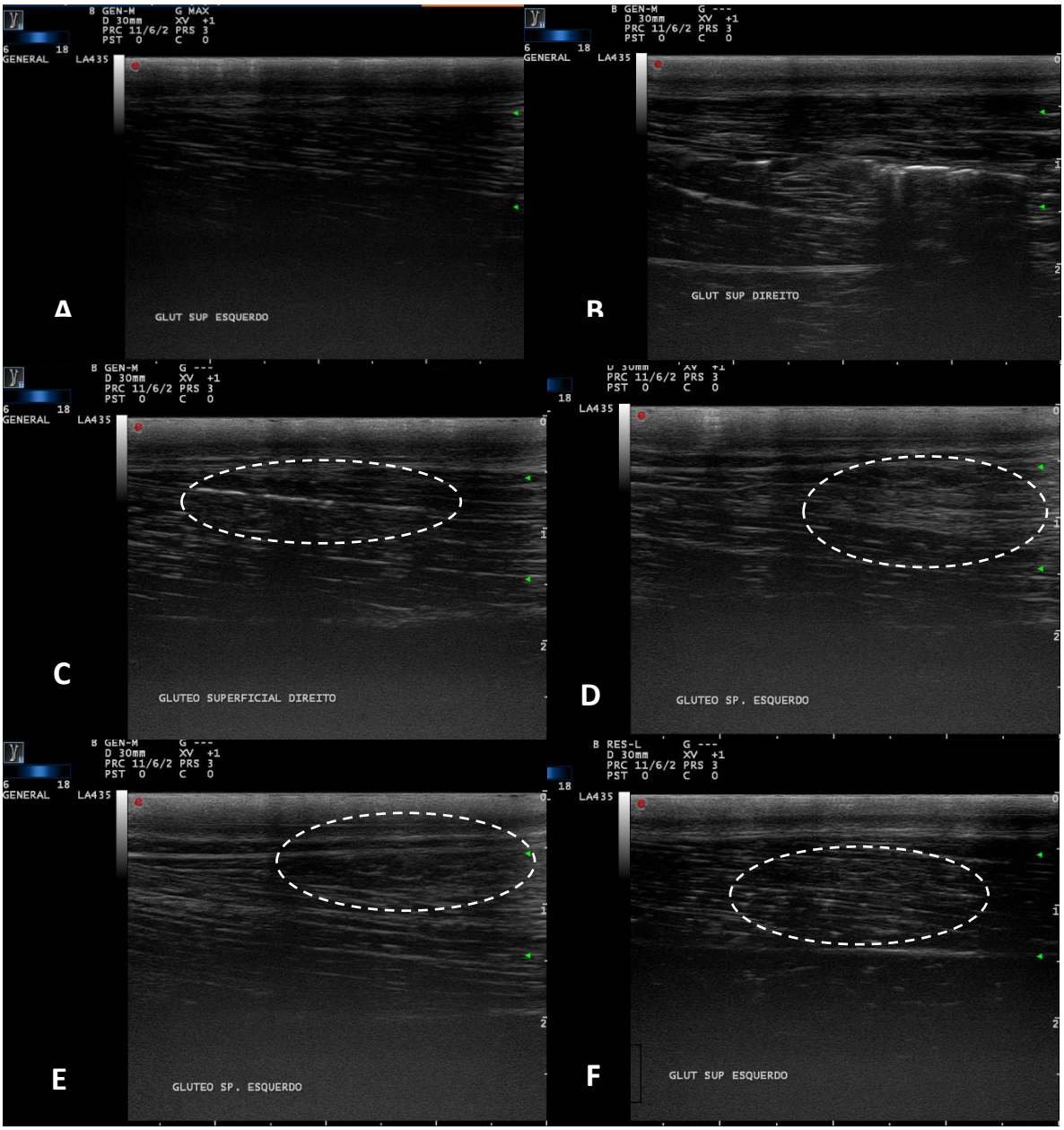

Figure 6: Ultrasonographic examination. (A) A normal superficial gluteo muscle; and (B) the moment of application. The presence of fluid injected between the muscle fibers can be observed, which caused expansion and separation of muscle fibers; (C) hyperechoic linear imaging between fibers (HYPERLI; dotted circle) suggestive of the presence of applied content between the fiber; (D) hyperechoic region/area (HYPER; dotted circle), suggestive of the presence of hematoma; (E) hypoechoic areas with a loss of parallelism between fibers (HYPO; dotted circle), suggestive of edema; (F) hyperechoic punctiform images (HYPERPI; dotted circle), suggestive of fibrin deposition.

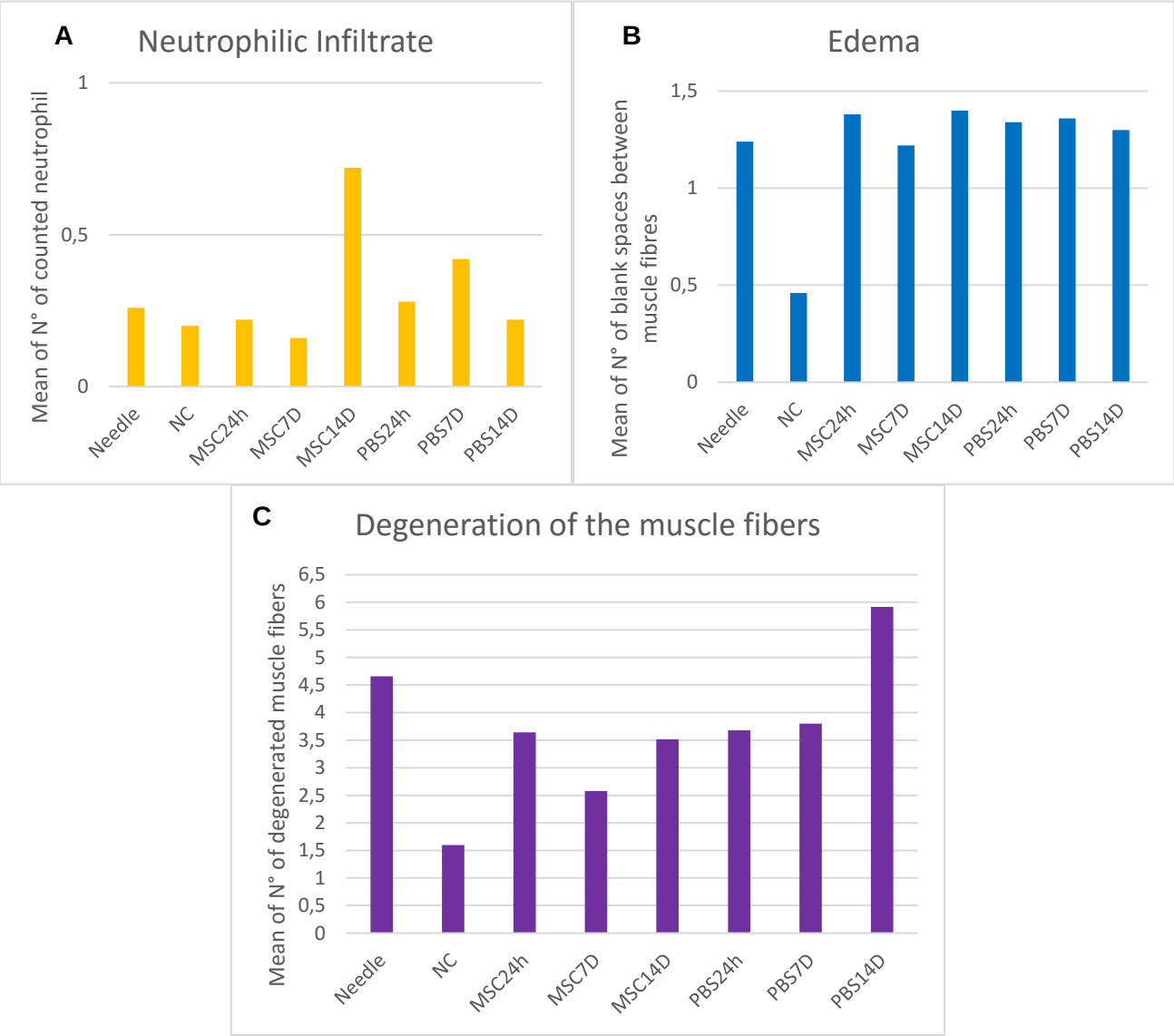

Figure 7: Histopathological avaliations. (A) Arithmetic mean of neutrophil counts in the 10 fields in each slide per animal per group. (B) The arithmetic mean of white fields, showing the dissociation of muscle fibers found in the 10 counted fields in each slide of each animal per group. (C) Arithmetic average of degenerate fibers found in the 10 counted fields in each slide of each animal per group.

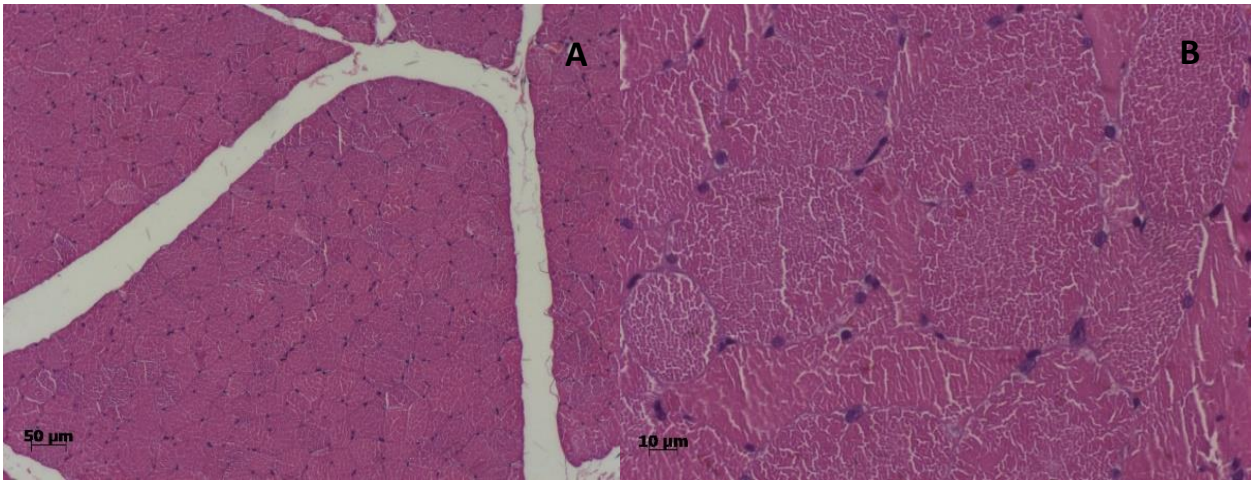

Figure 8: Histopathological exams of the superficial gluteus muscle of the NC group. The images show sections stained with hematoxylin & eosin (H & E). (A) Healthy muscle tissue, not subjected to any type of application (CN; 100×); (B) healthy muscle tissue, not subjected to any type of application (CN; 400×).

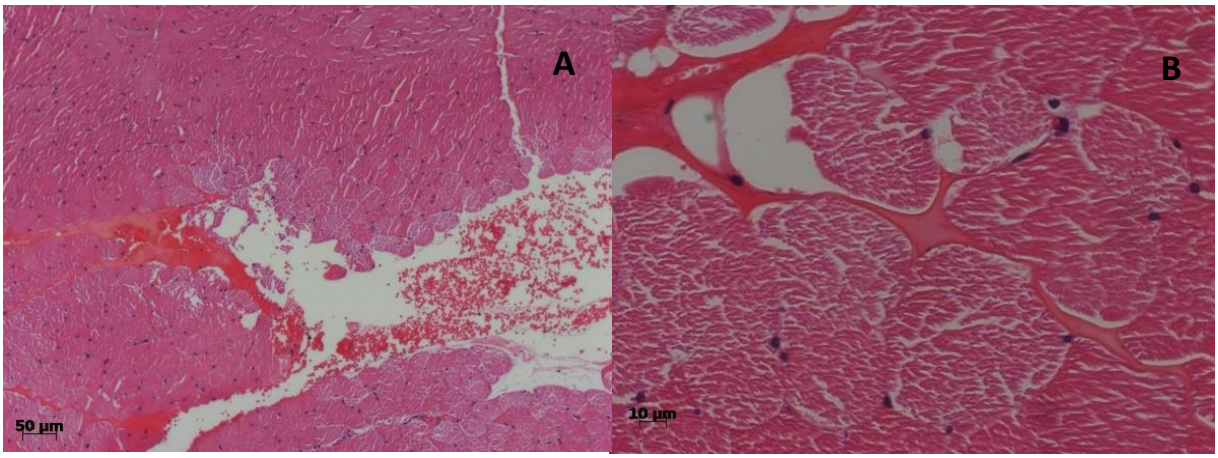

Figure 9: Histopathological exam of the superficial gluteal muscle in the needle group. The images show sections stained with hematoxylin & eosin (H & E). (A) Muscle tissue subjected to introduction of a needle without administering any content. We observed degeneration of muscle fibers and hemorrhage (100×); (B) Muscle tissue subjected to introduction of a needle without any content administration. We observed degeneration of muscle fibers (400×

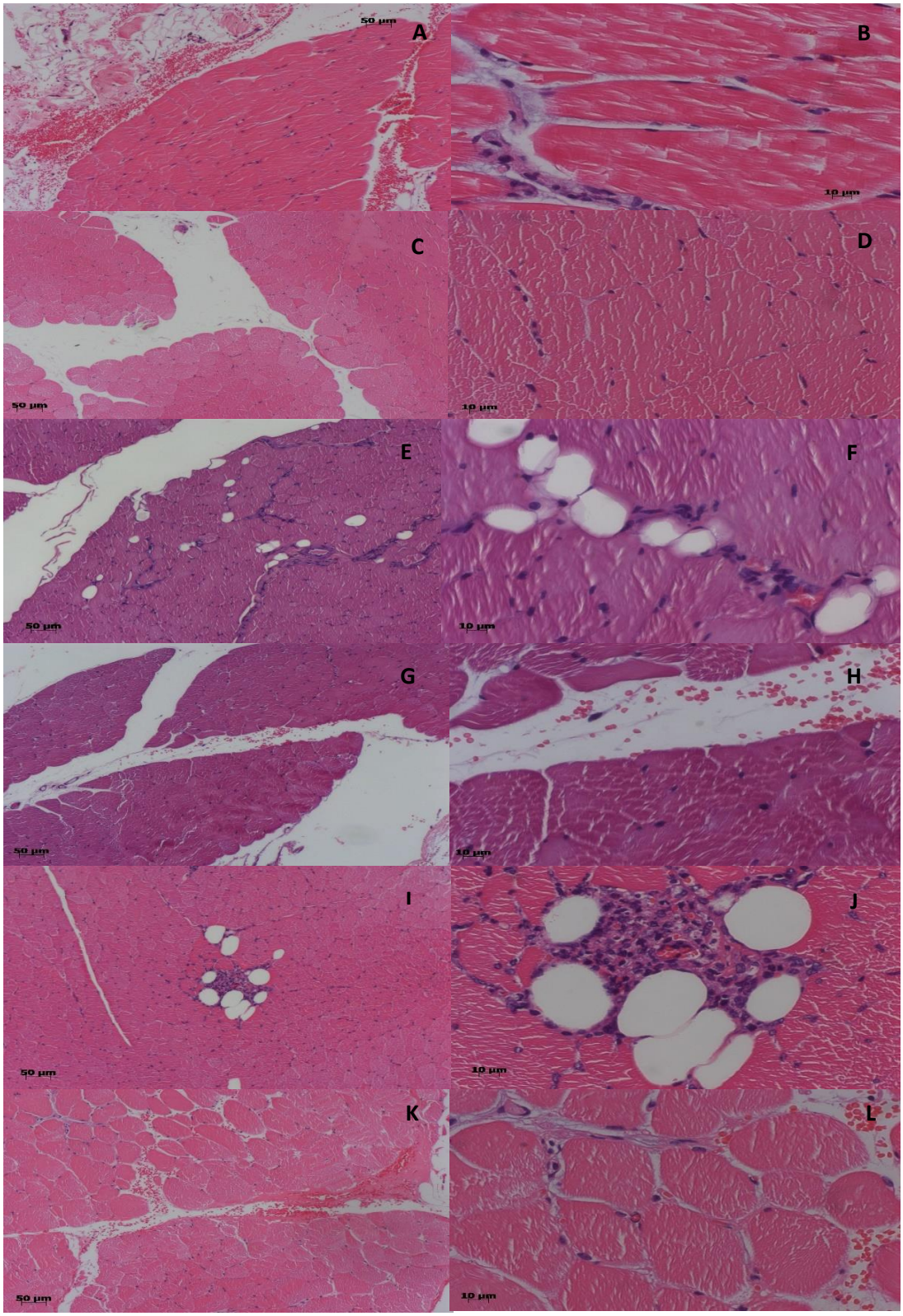

Figure 10: Histopathological exam of the superficial gluteal muscle of MSC24h, PBS24h, MSC7D, PBS7D, MSC14D and PBS14D groups. Images show sections stained with hematoxylin & eosin (H & E) for the following groups: **(A)** MSC24h (100×); **(B)** MSC24h (400×); **(C)** PBS24h (100×); **(D)** PBS24h (400×); (E) MSC7D (100×); (F) MSC7D (400×); (G) PBS7D (100×); (H) PBS7D (400×); (I) MSC14D (100×); (J) MSC14D (400×); (K) PBS14D (100×); (L) PBS14D (400×).

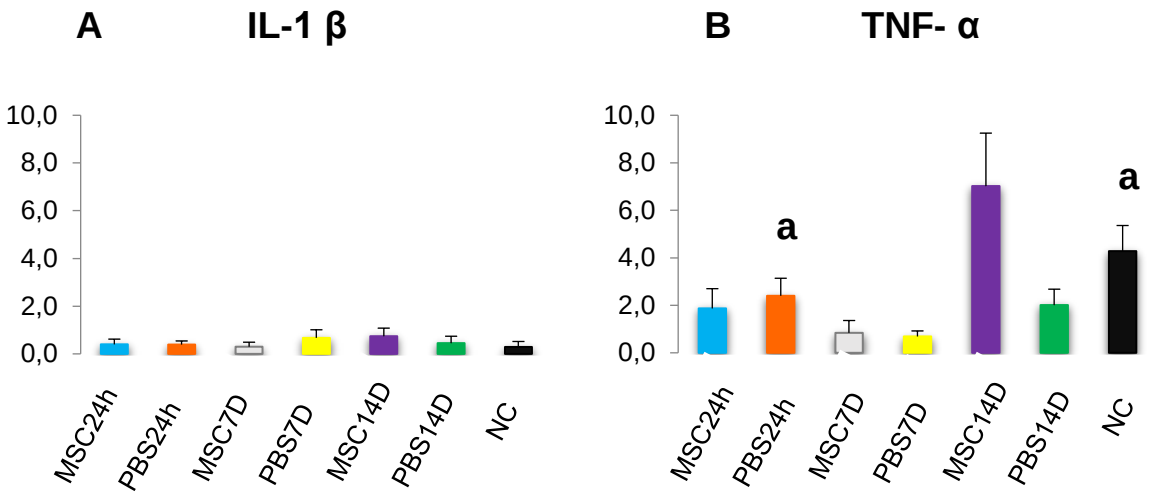

Figure 11: IL-1 β and TNF- α expression in the MSC24h, PBS24h, MSC7D, PBS7D, MSC14D and PBS14D groups and NC (healthy muscle). There was no significant difference in levels of IL-1β expression between the groups (P> 0.05).Those of TNF-α in all groups differed from the NC, except for the PBS24h group (P <0.05). Means followed by the same letter did not significantly differ by the Dunnett`s test (P> 0.05).

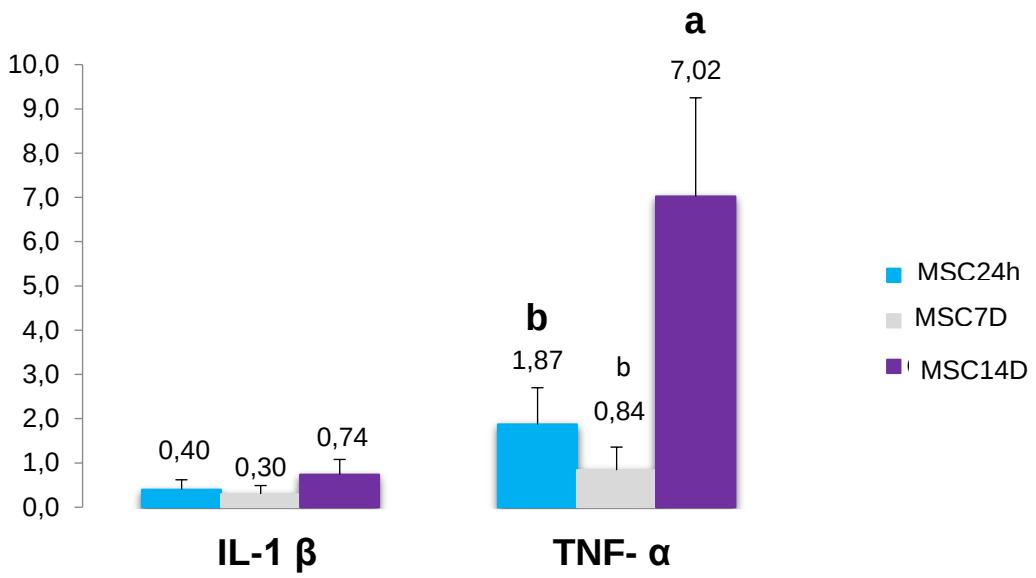

Figure 12: IL-1 β and TNF-α expression in the groups that received stem cells. Averages and standard deviation of the relative expression of IL-1 β and TNF-α in groups that received stem cells (MSC24h, MSC7D and MSC14D). Means followed by the same letter did not significantly differ by the Tukey test (P> 0.05).

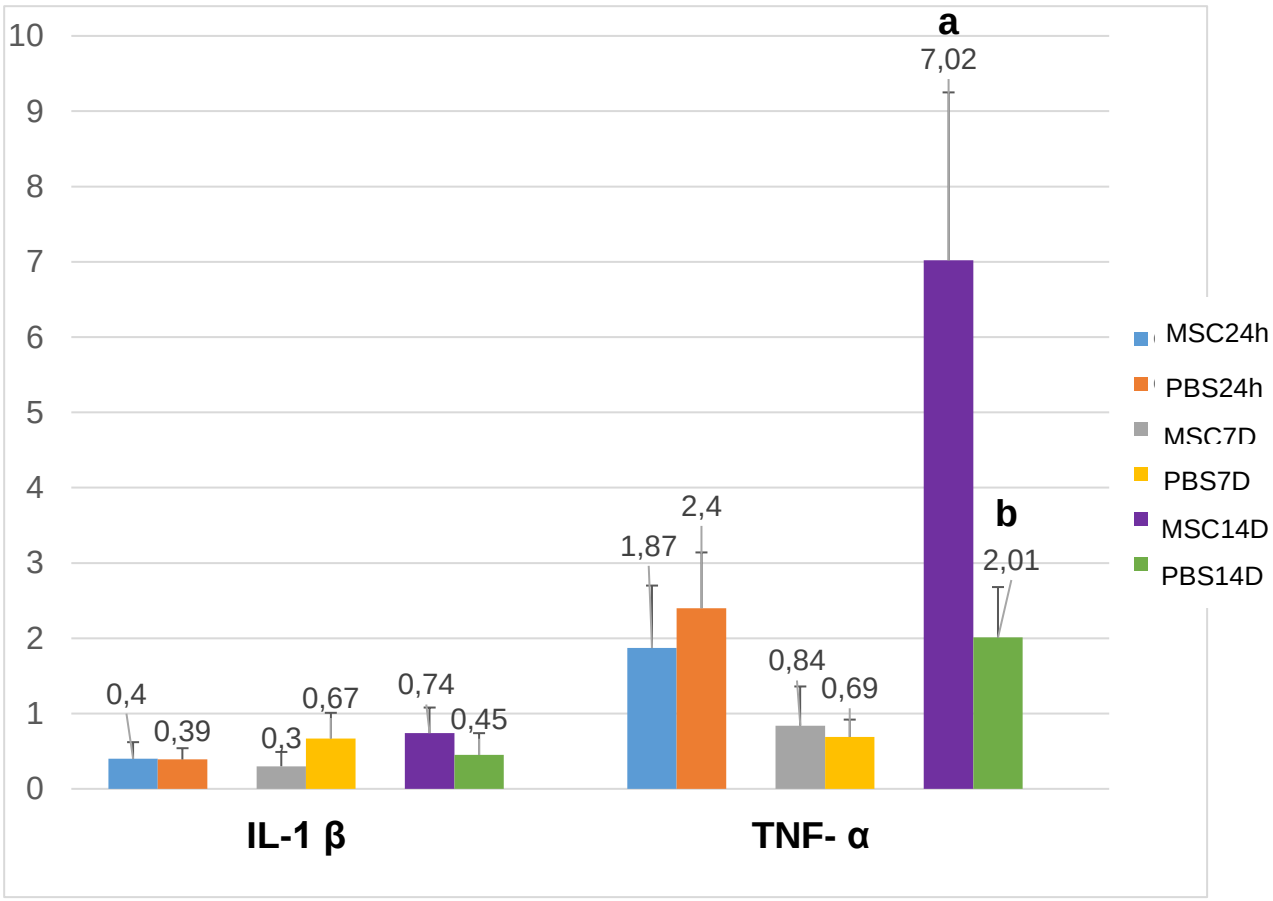

Figure 13: IL-1 β and TNF- α expression in groups that received stem cells and in controls. Mean and standard deviation of the relative expression of IL-1 β and TNF- α at all experimental groups, comparing groups subjected the application of stem cells and their respective controls (PBS application). Means followed by the same letter did not differ significantly by the Tukey test (P> 0.05).