UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

TRANSCRIPTOMA E CARACTERIZAÇÃO DO PERFIL PROTEÔMICO DE CÉLULAS-TRONCO MESENQUIMAIS DO TECIDO ADIPOSO DE MINI-HORSES (*Equus ferus caballus*)

ALICE PEREIRA MACIEL

Botucatu - SP
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ALICE PEREIRA MACIEL

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São Tomás de Aquino

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MACIEL, A. P. Transcriptoma e caracterização do perfil proteômico de células-tronco mesenquimais do tecido adiposo de mini-horses (*Equus ferus caballus*). Botucatu, 2019. p.62 dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista (Unesp).

As células-tronco mesenquimais extraídas da medula óssea ou tecido adiposo são utilizadas em abordagens terapêuticas, em vista das suas propriedades anti-inflamatórias, imunomoduladoras e regenerativas. Estas células são consideradas uma nova alternativa para o tratamento de enfermidades em diversas espécies, incluindo as dos equinos. Dessa forma, com o intuito de investigar a possível utilização das células estromais de mini-horses em tratamentos de cavalos, nesse estudo foi realizada a colheita, isolamento, expansão, caracterização imunofenotípica e diferenciação em duas linhagens mesenquimais: osteogênica e adipogênica de células estromais de mini-horses (*Equus ferus caballus*), objetivando a formação de um banco celular. Foi ainda realizada a análise transcricional comparativa dessas células, com as células estromais oriundas de cavalos (*Equus caballus*), produzidas e caracterizadas nas mesmas condições. Os parâmetros analisados foram o perfil de genes relacionados à angiogênese, regulação negativa da proliferação de células T, regulação negativa da ativação de células T, proliferação negativa de linfócitos reguladores, regulação negativa da proliferação de células mononucleares, regulação negativa da adesão célula-célula de leucócitos, regulação negativa da proliferação de leucócitos, regulação negativa da adesão célula-leucócito, regulação negativa da ativação de linfócitos. Também foram pesquisados os principais genes *up* e *down* regulados comparando mini-horses aos cavalos. Para formação do banco celular foram realizadas avaliações que comprovaram as características de células estromais nas células obtidas dos mini-horses, a avaliação imunofenotípica por citometria de fluxo demonstrou expressão alta dos marcadores CD90 (99,83 ± 0,28) e esperada do CD105 (38,66 ± 18,47) e expressão baixa dos marcadores CD34 (3,55 ± 1,14) e MHC II (4,21 ± 2,03). O potencial de diferenciação em linhagens adipogênicas in vitro foi observado

após o 14º dia através da visualização da deposição de gotículas lipídicas no citoplasma, e a linhagem osteogênica foi identificada após o 21° dia pela coloração positiva da matriz de cálcio extracelular. Ao final da avaliação transcricional, observamos um perfil de expressão gênica diferente entre os grupos, sugerindo que as diferenças entre os animais estudados se expande a mutação do gene Aggrecan que determina o nanismo. Apesar disso, os resultados indicam que as células estromais de mini-horse podem ser utilizadas como uma alternativa para o tratamento de lesões equinas com a vantagem de apresentarem uma maior expressão de genes relacionados à imunomodulação.

Palavras-chave: Cultivo celular, Diferenciação, Equinos, Gene, Terapia celular.

ABSTRACT

MACIEL, A. P. Transcriptome and protein profile characterization of mesenchymal stem cells from adipose tissue of mini horses (*Equus ferus caballus*). Botucatu, 2019. 62p. Thesis dissertation (Master degree) – School of Veterinary Medicine and Animal Science, Botucatu Campus, São Paulo State University (Unesp).

Mesenchymal stem cells extracted from bone marrow or adipose tissue are used in therapeutic approaches in view of their anti-inflammatory, immunomodulatory and regenerative properties. These cells are considered a new alternative for the treatment of diseases in several species. In order to investigate the possible use of mini-horses stromal cells in horse treatments, in this study was performed the collection, isolation, expansion, immunophenotypic characterization and differentiation in two mesenchymal lines were performed: osteogenic and adipogenic stromal cell mini-horses (*Equus ferus caballus*), aiming the formation of a cellular bank. We also performed the comparative transcriptional analysis of these cells, with stromal cells from horses (*Equus caballus*), produced and characterized under the same conditions. The parameters analyzed were the profile of genes related to angiogenesis, negative regulation of T cell proliferation, negative regulation of T cell activation, negative proliferation of regulatory lymphocytes, negative regulation of mononuclear cell proliferation, negative regulation of cell-cell adhesion leukocytes, negative regulation of leukocyte proliferation, negative regulation of cell-leukocyte adhesion, negative regulation of lymphocyte activation. We also searched the top 10 genes up and down regulated comparing mini-horses to horses. For cell bank formation tests were performed that demonstrated the characteristics of stromal cells in the cells obtained from the mini-horses, the immunophenotypic evaluation by flow cytometry showed high CD90 (99.83 ± 0.28) and expected CD105 (38.66 ± 18.47) and low expression of the CD34 (3.55 ± 1.14) and MHC II (4.21 ± 2.03) markers. The potential for differentiation in in vitro adipogenic lines was observed after day 14

by visualization of the deposition of lipid droplets in the cytoplasm, and the osteogenic lineage was identified after 21 ° day by the positive staining of the extracellular calcium matrix. At the end of the transcriptional evaluation, we observed a different gene expression profile between the groups, suggesting that the differences between the studied animals expands the Aggrecan gene mutation that determines the dwarfism. However, the results strongly indicate that ASCs from mini-horses can be used as an alternative to treat horse's diseases, with the vantages of presenting a higher expression of immunomodulatory genes.

Key words: Cell culture, Cell therapy, Differentiation, Equines, Gene.

CAPÍTULO I

1. INTRODUÇÃO

Os mini-horses (*Equus ferus caballus*) são integrantes do grupo de equídeos que englobam os cavalos (*Equus caballus*) e, os asininos ou jumentos (*Equus asinus*) (LESCHONSKI; SERRA; MENANDRO, 2008), que recentemente vem ganhando notoriedade em exposições e haras devido a sua inclusão no mercado pet. Sua conformação, semelhante a um cavalo em miniatura ocorre por alteração genética, devido à espécie ser oriunda do cruzamento de equinos de diferentes raças, resultando em fêmeas com altura máxima de 0,98 m e machos 0,93 m, que mesmo apresentando um pequeno porte, são considerados como uma raça de tripla aptidão, com a vantagem, que quando comparados a equinos de porte regular, necessitam de menores espaços e, consequentemente menores custos de criação, sem perder a resistência e rusticidade. Tais atribuições auxiliam na representatividade da raça, com significativo crescimento no Brasil (Associação Brasileira dos Criadores de Mini Horse, 2006).

A participação dos equídeos na economia nacional é representada através do complexo do agronegócio dos cavalos, a qual passou por um grande crescimento nos últimos anos, voltado especialmente para o público urbano, tanto no lazer como no esporte. Este crescimento tem resultado em maiores cuidados e gastos, que incluem despesas com medicamentos, ferragens, cosméticos e acessórios (MAPA, 2016). Perante isso, as pesquisas com equídeos no Brasil, assim como nos demais países, seguem relacionadas às perspectivas da indústria, abrangendo a produção, manejo, melhoramento genético, reprodução, clinica e cirurgia (ALMEIDA; SILVA, 2010), possibilitando o crescimento de diferentes áreas. Dentre estas, um grande avanço no segmento biomédico, que abrange a biologia celular e a medicina regenerativa, estimulando o conhecimento sobre as células-tronco (VERFAILLIE; PERA; LANSDORP, 2002).

As células-tronco mesenquimais vêm sendo muito estudadas tanto visando melhoria na qualidade de vida do animal, quanto ao procurar possíveis

tratamentos para enfermidades de difícil ou nenhum tratamento. Por serem de fácil obtenção e manipulação *in vitro*, pouco imunogênicas, possuírem habilidade de integração ao tecido hospedeiro, entre outros critérios, as células mesenquimais são consideradas grandes candidatas para aplicação em terapia celular (PITTENGER et al, 1999). Na espécie equina, tem-se relatos do uso terapêutico das células tronco mesenquimais principalmente oriundas da medula óssea (GUEST; SMITH; ALLEN, 2008) e tecido adiposo, apesar de já ter sido evidenciado o isolamento das células originarias do sangue (KOERNER et al, 2006), geleia de Wharton (ROMANOV; SVINTSITSKAYA; SMIRNOV, 2003) e de outros tecidos placentários (VERFAILLIE, 2002).

Considerando a ausência de publicações relacionadas ao cultivo de células estromais em mini-horses, e por estes estarem ganhando notoriedade no meio equino, o presente trabalho visa à coleta, isolamento e cultivo de células-tronco mesenquimais de mini-horses, utilizando como fonte o tecido adiposo, para comparação de suas características *in vitro* e de seu transcriptoma com as células-tronco de equinos produzidas utilizando o mesmo protocolo.

1.1 Objetivo Geral

- Avaliar o isolamento de células-tronco mesenquimais, também chamadas de células estromais mesenquimais (MSCs), obtidas do tecido adiposo de mini-horses (*Equus Ferus caballus*), realizando a expansão *in vitro*, caracterização e diferenciação, bem como o transcriptoma.

1.2 Objetivo Específico

- Comparar o transcriptoma das células estromais mesenquimais do tecido adiposo de cavalos (*Equus caballus*) e mini-horses (*Equus ferus caballus*).

2. REVISÃO DE LITERATURA

2.1. CÉLULA-TRONCOS

As células-tronco são células não especializadas (BYDLOWSKI; DEBES; MASSELLI, 2009), precursoras, com a capacidade de autorrenovação e diferenciação, que podem formar diferentes tipos teciduais (WATT; HOGAN, 2000). Sua definição é baseada em alguns princípios, como a capacidade de: 1) autorrenovação, ou seja, a possibilidade de originar uma célula-filha com características idênticas às de sua progenitora; 2) diferenciação de uma única célula em multilinhagem; 3) reconstituição, pela produção de células funcionais in vivo em um determinado tecido (WEISSMAN, 2000). Devido a sua origem e capacidade de diferenciação, podem ainda ser agrupadas como células-tronco embrionárias (pluripotentes) e células-tronco de adultos (somáticas, multi ou unipotentes) (ZAGO; COVAS, 2004).

Células totipotentes ou embrionárias são as primeiras células originadas na fertilização, ou seja, das primeiras divisões dos oócitos após a fusão com o espermatozoide. Esse tipo celular pode originar qualquer tipo de célula e tecido. As linhagens multipotentes ou pluripotentes, dão origem a quase todos os tecidos, com exceção da placenta e os anexos embrionários. Já as oligopotentes e unipotentes, são respectivamente aquelas que conseguem se diferenciar em poucos tecidos e as que produzem apenas um tipo celular, porém devido a sua capacidade de autorrenovação são consideradas células-tronco (MARQUES 2006).

Apesar do grande potencial oferecido pelo uso das células pluripotentes, essas células não são utilizadas clinicamente, devido ao risco de formação de teratomas (SAITO; MINAMIHASHI; UGAI, 2006). Contudo, as células-tronco mesenquimais (CTMs), obtidas de tecidos adultos, devido a sua capacidade como precursoras multipotentes de tecidos conectivos, como o músculo, e por possuírem capacidade de autorrenovação e diferenciação, tem se mostrado

relevantes na medicina regenerativa em diversas espécies, incluindo a espécie equina, sendo as células de eleição para o tratamento de lesões tendíneas de equinos (TAYLOR; SMITH; CLEGG, 2007).

2.2. CÉLULAS-TRONCO MESENQUIMAIS OU CÉLULASESTROMAIS MESENQUIMAIS (CTMs)

Há mais de quatro décadas Friedenstein et al. (1968), observou células isoladas da medula óssea, as quais, foram descritas como uma população de células-tronco, multipotentes, de formato fusiforme, chamadas de unidade formadora de colônia de fibroblastos (CFU-F). Estas células-tronco adultas foram classificadas como multipotentes, por serem indiferenciadas, e apresentarem a capacidade de renovação e produção de tipos especializados de tecidos mesenquimais (SLACK, 2000), tendo recebido posteriormente o nome de células -tronco mesenquimais (Caplan et al., 1991) Sua possibilidade de isolamento e manipulação representa um avanço promissor no reparo tecidual e regeneração da engenharia dos sistemas, órgãos e tecidos (BRUDER et al, 1998).

As funções primordiais dessas células, quando em um organismo, são a manutenção e reparação de tecidos. As CTMs podem ser obtidas de diferentes tecidos, como a córnea e retina (SLACK, 2000), músculo esquelético e derme (YOUNG et al, 2001), hematopoiético (CABRITA et al, 2003), cordão umbilical (DIMITRIOU et al, 2004), liquido amniótico (DE COPPI et al, 2007), sangue menstrual (ALCAYAGA et al, 2015), medula óssea (CAMPAGNOLI et al, 2001) polpa dental (MADAN; KRAMER, 2005) e tecido adiposo (ZUK; ZHU; MIZUNO, 2001).

O tecido adiposo (ZUK; ZHU; MIZUNO, 2001), em comparação com a medula óssea, possui maior facilidade de colheita, maior índice de proliferação in vitro e cerca de 2% do total obtido de cada amostra são de CTMs, enquanto na medula óssea esse valor decresce para aproximadamente 0,002% (DAHLGREN, 2009).

O tecido adiposo é classificado em dois tipos, o marrom e o branco. Em humanos adultos o tecido adiposo marrom é considerado escasso, enquanto que os depósitos de tecido adiposo do tipo branco estão distribuídos por todo o corpo, envolvendo órgãos e estruturas internas, sendo subdividido em tecido adiposo subcutâneo e visceral. Possuem adipócitos e outros tipos celulares, como leucócitos, macrófagos e células-tronco (FRUHBECK et al, 2001). Usualmente, para aquisição de células provenientes desse tecido, é colhida uma amostra da região supragluteal ou base da cauda, o tecido adiposo subcutâneo (MAIA et al, 2009), o qual é utilizado para o processo de isolamento de células nucleadas, feito pela digestão com colagenase, seguido por uma série de centrifugações, com o intuito de separar células específicas (ZUK; ZHU; MIZUNO, 2001). Por incluírem diversos progenitores celulares, as células obtidas do tecido adiposo são consideradas uma população heterogênea (GOODSHIP, 2004), composta por células endoteliais, epiteliais, fibroblastos, mastócitos, pré-adipócitos e CTMs (ZUK; ZHU; MIZUNO, 2001). Esta mistura de células é chamada de fração estromal do tecido.

Independente da fonte e espécie estudada, como cães (OLIVEIRA et al, 2010), coelhos (EURIDES et al, 2010), gatos (MULLER et al, 2009), humanos (NISHIKAWA; GOLDSTEIN; NIERRAS, 2009) ovinos (MRUGALA et al, 2008), ratos (BAKSHI et al, 2006) ou equinos (RICHARDSON et al, 2007). A International Society for Cellular Therapy, determina três requisitos básicos para que uma população de células seja classificada como CTMs (HORWITZ et al, 2005).

Primeiramente, as células devem ser isoladas de uma população de células mononucleares ou estromais com base à sua aderência seletiva, em cultura, à superfície do plástico. Segundo deve ocorrer expressão de CD73, CD90 e CD105, e que CD34, CD45, CD14, ou CD11b, CD79, ou CD19, MHC II e HLA-DR não sejam expressos em mais de 5% das células em cultura. E, as células devem ter a capacidade de diferenciação in vitro em osso, gordura e cartilagem (HORWITZ et al, 2005).

Contudo, a expressão positiva de marcadores pode ser resumida com a utilização do CD105 e CD90, desde que marcadores hematopoiéticos não sejam expressos, e sejam comprovados longos períodos de aderência celular ao plástico em cultura e a realização de diferenciação em no mínimo duas linhagens distintas (HORWITZ et al.,2005).

A interação entre as CTMs e as demais células do organismo, se dá pela alta expressão de moléculas bioativas, por elas produzidas, como a liberação de fatores de crescimento e citocinas (HUSS, 2000), que devido as suas atividades autócrinas e parácrinas, além de exercerem ação antiapoptotica, reparação endógena e ação pró-angiogênica, contribuem para os efeitos terapêuticos observados com a utilização das células (GNECCHI et al, 2008).

Outra característica importante é a capacidade de homing, descrito como a capacidade das CTMs serem atraídas para a rede vascular de um tecido e migrarem pelo endotélio para determinados órgãos e tecidos em resposta a produção de fatores de quimiotaxia (KARP; LENG, 2009).

2.3. ATIVIDADE IMUNOMODULADORA DAS CTMs

Os mecanismos de respostas ao dano tecidual envolvem a interação de diferentes tipos celulares, humorais e elementos do tecido conjuntivo, a fim de limitar a invasão tecidual e proporcionar o reestabelecimento de sua integridade (EMING; KRIEG; DAVIDSON, 2007).

Diversos trabalhos já demonstraram que as CTMs tem a habilidade de exercer efeitos regulatórios e supressivos sobre a resposta imunológica inata e adaptativa (COULSON-THOMAS et al., 2016). As CTMs têm a capacidade de formar nichos em espaços perivasculares e respondem a citocinas liberadas durante o processo inflamatório, sendo ativadas principalmente por IFNγ, IFNα e IL-1β (LEE; SONG, 2018). Acredita-se que citocinas atraiam as CTMs para as áreas lesadas, resultando na migração para esses locais com o intuito de estimular a reparação e a cicatrização tecidual (GLENN; WHARTENBY, 2014).

A ação regulatória das CTMs se dá sobre macrófagos (SONG; XIE; LU, 2015), neutrófilos (KHAN et al, 2015), células dendriticas (MOHAMMADPOUR et al., 2015) e linfócitos “natural killers” (QU et al., 2015), modulando desta forma a primeira linha de defesa do organismo. Além disso, as CTMs também regulam os linfócitos T e B, ou seja, o sistema imune adaptativo. As CTMs inicialmente promovem a ativação de linfócitos T reguladores (GHANNAM et al., 2010) e inibem a proliferação de linfócitos B, diferenciação de plasmócitos e portanto a produção de anticorpos (ROSADO et al., 2015).

A inibição da proliferação de células do sistema imune promovida pelas CTMs, ocorre por paralisação da fase do ciclo celular G0/G1 (ZAPPIA et al, 2005), mas sem indução de apoptose (FRANQUESA et al, 2012). Por possuírem capacidade de modulação da ativação e proliferação das células T reguladoras (KRAMPERA et al., 2003), as CTMs modulam efeitos imunorreguladores, desempenhando um papel fundamental na transição da imunidade inata à adaptativa (LO et al, 1999).

2.4. EQUINOS E MINI-HORSE

As CTMs tem despertado interesse para o tratamento de enfermidades de equinos, devido a apresentação de resultados eficazes em injúrias (VIDAL et al., 2007), como lesões tendíneas (FORTIER; TREVIS, 2011), recuperação de cartilagem (FORTIER; SMITH, 2007) e endometrite crônica regenerativa (PAVÃO, 2013), recentemente tem-se pesquisado a utilização do meio condicionado, oriundo dessas células como alternativa de tratamentos (LANGE-CONSIGLIO et al, 2013).

A utilização de mini-horses tem sido evidenciada na pesquisa como alternativa ao uso de cavalos (MAZZO, 2018), a American Mini- Horse Association (AMHA), comparou os custos da criação do que eles chamaram de equinos em “tamanho completo”, em relação aos mesmos itens para mini-horses, revelando que inicialmente apesar do tempo de vida útil ser o mesmo para ambos, o preço para aquisição dos mini-horses é significativamente inferior, e os custos com alimentação dos equinos é em média 6 vezes maior,

além dos custos com tratador e treinador que podem ser duplicados, também foi observado que a área com capacidade para comportar 3 mini-horses equivale ao espaço necessário para um cavalo.

O tamanho dessa espécie ocorre devido ao nanismo, uma malformação congênita, cuja transmissão na maioria dos casos ocorre de forma hereditária (THOMPSON, 2007), autossômica recessiva (OSINGA, 2000). No estudo realizado por Eberth (2003), foi observado que as formas mais frequentes do aparecimento de nanismo em mini-horses, são por consequência de mutações no gene Aggrecan (ACAN), pois este se apresenta de quatro formas, (D1, D2, D3 e D4), porém, a forma D1 em combinação com qualquer outro alelo mutante tem característica letal.

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CAPÍTULO II

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Comparation of the transcriptome profiling of mesenchymal stromal cells from mini-horses (Equus Ferus caballus) and horses (Equus caballus).

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Abstract

Background

Although mini-horses (*Equus ferus caballus*) are most similar to horses (*Equus caballus*), differences still exist in their epigenetic landscape and transcribed genes, to be studied. The objective of this study was to investigated whether adipose-derived stromal cells (ASCs) from horses can be replaced by ASCs from mini-horses to be used in the treatment of equine diseases. Thus, the Equine and Mini-horse ASCs gene expression was compared using transcriptomics analyses, with an emphasis on angiogenesis and the immunomodulatory activities.

Methods

ASCs of Mini-horses were collected, expanded *in vitro* and characterized, as well as evaluated in comparison with ASCs from horses for phenotype, and transcriptome analysis.

Results

ASCs from mini-horses, were highly proliferative and fit the minimal criteria of multipotent stromal cells, been positively label and for CD90 and CD105, and with negative expression of CD34 and MHC II. Moreover, they present capacity of differentiation into adipogenic and osteogenic lineages. In the functional analysis of the transcriptome 20.777 genes were identified, but only 237(1,14%) presented a significant difference between the mini-horse ASCs in relation to the equine ASCs. Genes up regulated in the mini horse were related to negative regulation of several immune cell. On the other hand, down regulated genes were related to angiogenesis and negative regulation of cell growth and motility.

Conclusion

Findings suggest that the differences in the transcriptome of ASCs from mini-horses and horses is very small suggesting that ASCs from mini-horses can be used for horse cell therapy.

Keywords

Adipose-derived stromal cells, Cell therapy, Differentiation, Equine, Gene, Stem cell.

Background

Since they were first isolated, from bone marrow, by Friedenstein et al. [1] in 1968, mesenchymal stem cells (MSCs) have become a subject of experimental studies. Besides being isolated from the bone marrow, cells with characteristics similar to bone marrow (BM-MSCs) have been found in most mammalian tissues examined [2,3]. The adipose tissue, is extremely rich in MSCs being considered the most abundant source [4], with the advantage of been also easer to collect [5]. The most popular method for separating MSCs relies on isolation of stromal cells from various tissues, with or without protease treatment. When in culture, fibroblast-like cells adhering to the culture plate surface are selected and non-adherent floating cells are removed. Due to the heterogeneity of the population, MSCs isolated by this method are suggested to be termed Mesenchymal Stromal Cells, not Mesenchymal Stem Cells, although both have the same acronym, MSC. However, when adipose tissue is used these cells may also be referred to as adipose-derived stromal cells (ASCs) [6].

Heterogeneous populations of MSCs are fibroblast shaped cells present in perivascular location of most tissues. These cells secrete a wide variety of growth factors cytokines and adhesion molecules by which they interact with the immune cells in inflamed and damaged tissues leading to tissues regeneration via positive paracrine effects [7]. MSCs can modulate functions of activated T cells [8,9], B cells [10], NK cells [11], dendritic cells (DCs) [9], and macrophages [12], creating an environment suitable to immune response modulation. Another important characteristic is that MSCs are poorly recognized by the host immune system been able to escape the immune system recognition mechanisms which facilitate their use in cell therapies [13].

The MSCs present various forms of applications [14,15] in cell therapy [16]. When administered, those cells have the ability to migrate to the niches in perivascular spaces attracted by cytokines secreted by the inflammatory cells such as IFNγ, IFNα e IL-1β [7]. It is believed that those cytokines attract MSCs to the injured areas, resulting in migration to these sites in order to stimulate repair and tissue healing [17,18]

MSCs therapeutic use was described in several species to treat degenerative diseases [19,20]. In equines its use has been associated mainly with tendinous lesions [21]. However, due to their unique immunomodulatory properties, combined with their other tissue regeneration properties make it possible to use them also in other diseases. Several studies revealed that MSCs isolated from different sources are not exactly the same and the highly heterogeneous populations of cells are dramatically affected by various extrinsic and intrinsic factors such as species, tissue of origin, animal age, and others [22].

Although mini-horses (*Equus ferus caballus*) are most similar to horses (*Equus caballus*), differences still exist in their epigenetic landscape and transcribed genes to be studied. In the present study, we investigated whether ASCs from horses can be replaced by ASCs from mini-horses to be used in the treatment of equine diseases. Thus, we compare systematically the expression of genes related to angiogenesis and the immunomodulatory activities of these cells using transcriptomics analyses.

Methods

Ethics statement

All stages of this experiment were approved by the Ethics Committee on Animal Use of São Paulo State University (UNESP) – Botucatu (Protocol 030/2019-CEUA) and conducted in accordance with the Ethical Principles in Animal

Experimentation.

Animals and ASCs characterization

Ten adult male mini-horses, clinically healthy and aged between 2,5 –7 years old, were selected for adipose tissue harvest. The tissue was isolated, cultured and in the second passage (P2) a sample of cells was used for characterization by surface marker expression (immunophenotype) and evaluation for differentiation potential into two mesenchymal lineages:

adipogenic and osteogenic. Another sample was used for the transcriptome analysis.

Isolation, expansion and cryopreservation of mesenchymal stem cell by adipose tissue of mini-horses

Collection of adipose tissue was performed with the animals in station, following the anesthetic protocol used by Bravo et al. [23], the incision of approximately 4 cm was performed in the region of the paraxial caudodorsal gluteal region (tail base) and the adipose tissue layer was isolated between the skin and the musculature. The samples were individually stored in HBSS solution (ThermoFisher Scientific), plus 1% penicillin/streptomycin (ThermoFisher Scientific) and 1,2% amphotericin B (ThermoFisher Scientific).

The sample was washed 4 times, first with alcohol and then with HBSS/amphotericin/penicillin. After the washes, samples were transferred to a Petri dish and mechanically fractioned using two scalpel blades n° 24. Then, the samples were added of (0.04%) type 1 collagenase (Sigma) at 37,5°C for 30 min. Subsequently, the solution was filtered through a 70-micrometer filter, and centrifuged thrice at 340g for 10 min. The supernatant was discarded and samples were cultured with culture medium containing DMEM low glucose/F12 (ThermoFisher Scientific) plus 1% penicillin/estreptomicin and 1,2% anphotericim B in 25cm² culture flasks, for 5 days, without manipulation in a humidified atmosphere containing 95% air and 5% CO2 at 37,5°C. After this time, the culture medium was changed every 48 hours until a cellular confluence of ≥ 90%.

When a confluence of ≥ 90% was reached the trypsinization was accomplished. For this, 3 mL of heated Tryple® was added per flasks, kept at 37,5°C for 4 minutes and centrifuged at 940xG for 10 minutes. Posteriorly, the cell pellet was ressuspended in culture medium, and cultured into two 75-cm² flaks in the same conditions as described above. The cryopreservation protocol used was similar to that performed by Mitchell et al [24], using 90% FBS and 10% DMSO. The samples were reconstituted using the protocol previously described by Chaytor et al [25]. Just after thawing, the viability test was estimated with Trypan Blue (ThermoFisher Scientific). .

Immunophenotypic characterization and differentiation potential

Immunophenotypic analysis was performed at the end of secondary culture according to the technique described previously by Maia et al., 2015 [25], using a Fortessa LSR Cytometer (Becton Dickinson and Company, USA) and monoclonal mouse anti-rat CD90 (clone OX7, 1: 100, Caltag Laboratories, USA), mouse monoclonal CD105 (clone SN6, 10:100, abcam), mouse anti-human CD34 (clone 581/ CD34, 1:50, Becton Dickinson and Company, USA) antibodies labeled with fluorescein isothiocynate (FITC) and mouse anti-horse MHC de classe II (clone CVS20, 1:100 AbD Serotec, UK). During analysis, 10,000 events were recorded.

For osteogenic and adipogenic assay the protocol described by Maia et al, [26] was used. After reaching confluence in the secondary culture, ASCs were trypsinized and seeded at a density of 2 × 105 ASCs/well, in six-well plates (Sarstedt, USA). After six days, the maintenance medium was removed and media for osteogenic or adipogenic differentiation Stem- Pro (ThermoFisher) were added to the subcultures, in duplicate, according to the manufacturer's recommendation with modifications. The media were changed every 3 days and confirmation of osteogenic and adipogenic differentiation was obtained, respectively, by demonstrating the deposition of a calcium-containing matrix using the histological method of staining with 2% Alizarin red, pH 4.2 and the presence of intracytoplasmic lipid droplets by staining with 0.5% oil red O (Sigma-Aldrich Corp).

Equine ASCs

The horses stem cells were isolated, cultivated and characterized under the same conditions and provided by the cell bank of the laboratory.

RNA isolation, quality control and library preparation sequencing

Total RNA was extracted from three samples of ASCs mini-horses and ASCs horses cryopreserved using the TRIzol® reagent protocol according to

the manufacturer's instructions (TermoFisher Scientifc, Waltham, USA). RNA concentrations were quantifed in each sample using a QubitTM 2.0 fuorometer (TermoFisher Scientifc, Waltham, USA). The levels of RNA degradation were assessed by a 1% agarose gel. cDNA libraries were constructed with 200ng of total RNA using the SureSelect Strand Specifc RNA Library Prep Kit (Agilent Technologies, Santa Clara, USA) following the manufacturer's instructions. Library products were then sequenced using an Illumina Nextseq platform (Illumina, San Diego, USA) on a single-end 150bp run.

RNA-Seq data processing and diferential gene expression analysis

A CLC Workbench version 7.01 platform was used to remove the adapter and assess the reads quality from the raw reads. The same platform was used to subject reads to mapping to the reference and NCBI Cab3. Differential expression analysis was performed with DESeq2 package version 1.24.012. Genes presenting an adjusted p-value lower than 0.05 were considered as significantly regulated. Principal component analysis (PCA) data visualization and plotting were performed in R using the pcaExplorer package13. Clustering analysis was performed using Euclidean distances and complete linkage method and for gene ontology analysis, was used gProfiler;

Results

Cell adhesion

After five days of isolation from the adipose tissue all to the mini horse ASCs cultured adhered to the flask (Figure 1a), presenting fibroblastoid morphology (Figure 1b). After around fifteen days in average 90% cell confluence was reach (Figure 1c).

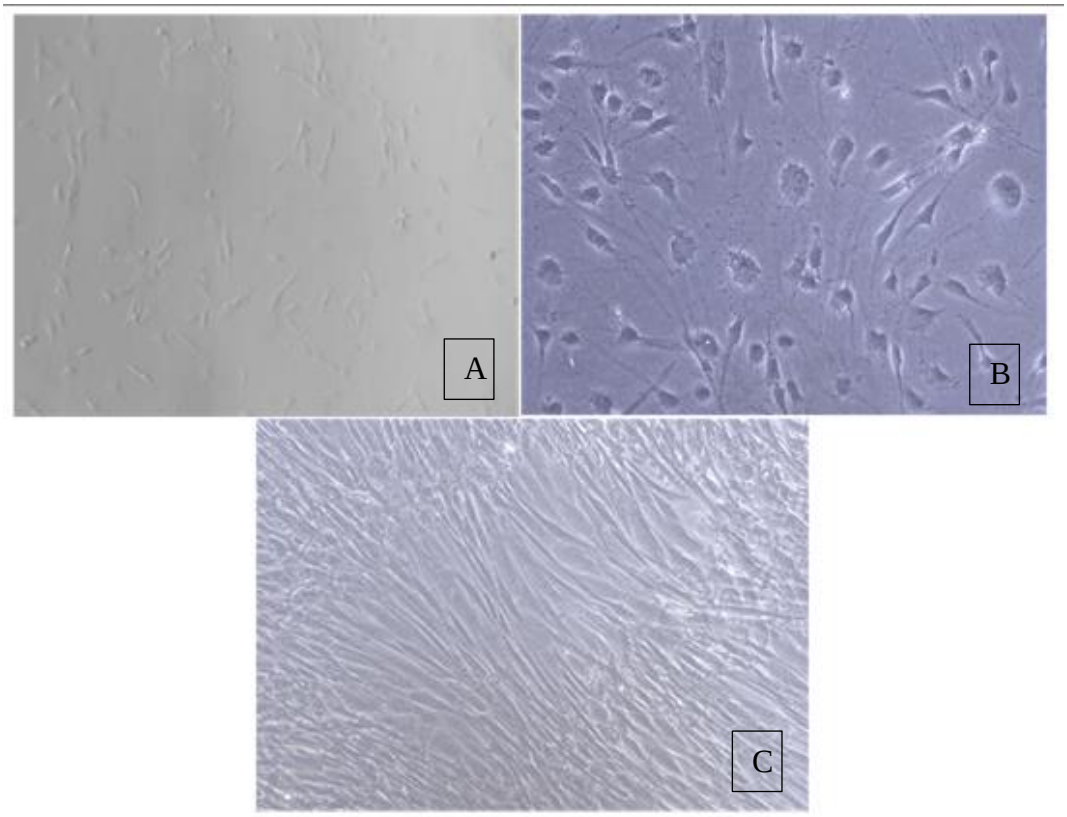

Figure 1. ASCs during cell culture (A) five days of aderenc (B) fibroblastoid morphology (C) with ≥90% confluence fifteen days. (Objective 20).

Surface marker expression

Immunophenotypic evaluation at P2 demonstrated mini-horse ASCs with a high expression of CD90 marker (99.83± 0,28) and a normal expression of CD105 (38,66 ± 18,47). Moreover, a low expression of CD34 (3,55 ± 1,14) and MHC II (4,21 ± 2,03) markers was also observed. CD expression under 5% was considered negative.

Differentiation into adipogenic and osteogenic lineages

Differentiation potential into adipogenic lineages was observed after 14th days of culture through visualization of the increase in lipid droplets in the cytoplasm of ASCs using 0.5% Oil Red staining (Figure 2A and 2B). The osteogenic lineage was identified after 21th days by positive staining of the extracellular calcium matrix using 2% Alizarin Red S staining (Figure 2C and 2D).

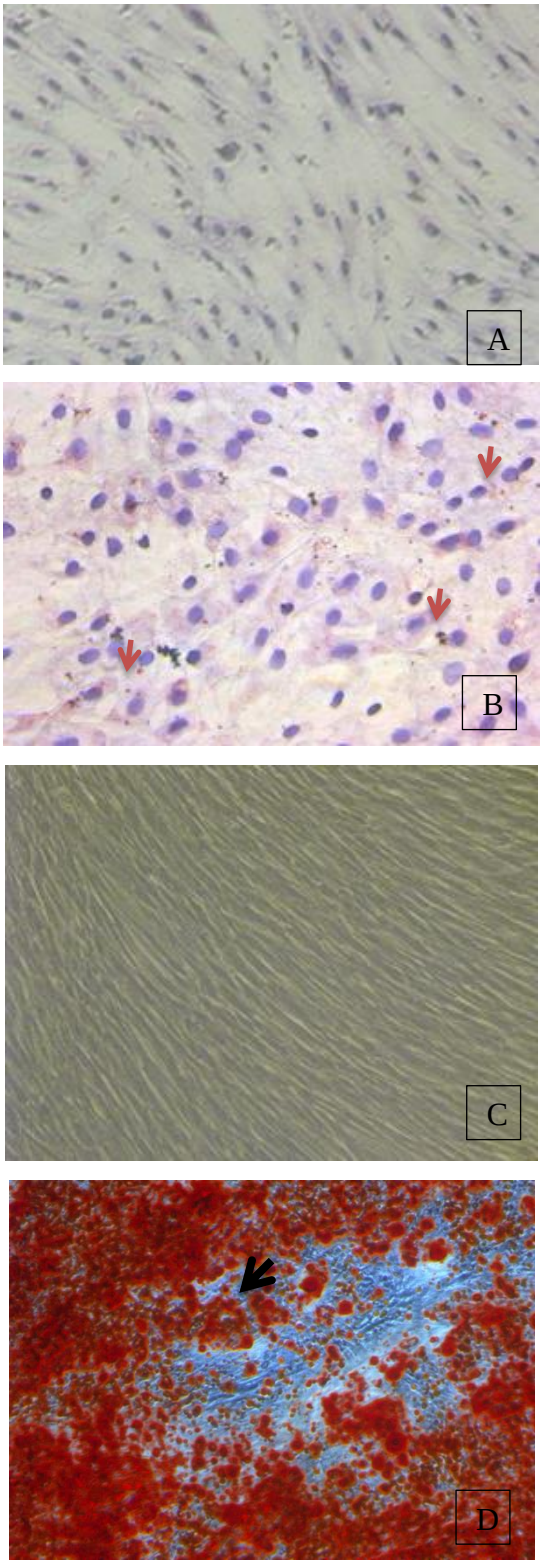

Figure 2. Differentiation potential of ASCs from mini-horses into two lineages. (A) ASCs showing control group for coloring Oil Red. (B) ASCs stained with Oil Red showing the intracytoplasmatic lipid droplets the adipogenic lineage. (C) ASCs showing control group for without coloring Alizarin Red (D) ASCs after osteogenic differentiation stained with Alizarin Red showing matrix calcium formation. (obj. 20x).

Transcriptome analysis

The transcriptome analysis was performed in order to evaluate the similarities and differences between mini-horses and horses ASCs. Transcriptome principal component analysis (PCA) indicated the presence of two distinct groups based on founded genes (PC1 + PC2 > 50% - Figure 3).

The sequencing identified a total of 20.777 transcripts (additional file 1) witch were classified based on abundance. The transcripts were analyzed for identification according to their statistical significance and biological role (table 2). When using Padj <0.05 we could observe that 237(1,14%) genes presented a significant difference between the mini-horse ASCs group in relation to the equine ASCs control group (Figure 4).

Differentially expressed genes were extracted from results of transcriptome analysis including the top over expressed genes showed on table 1 and the lower up-regulated genes present on table 2. Among the genes differentially expressed in the mini horse, angiogenesis, axon guidance, FGF signaling, inflammation mediated by chemokines and cytokines and integrin signaling were the most frequent pathways.

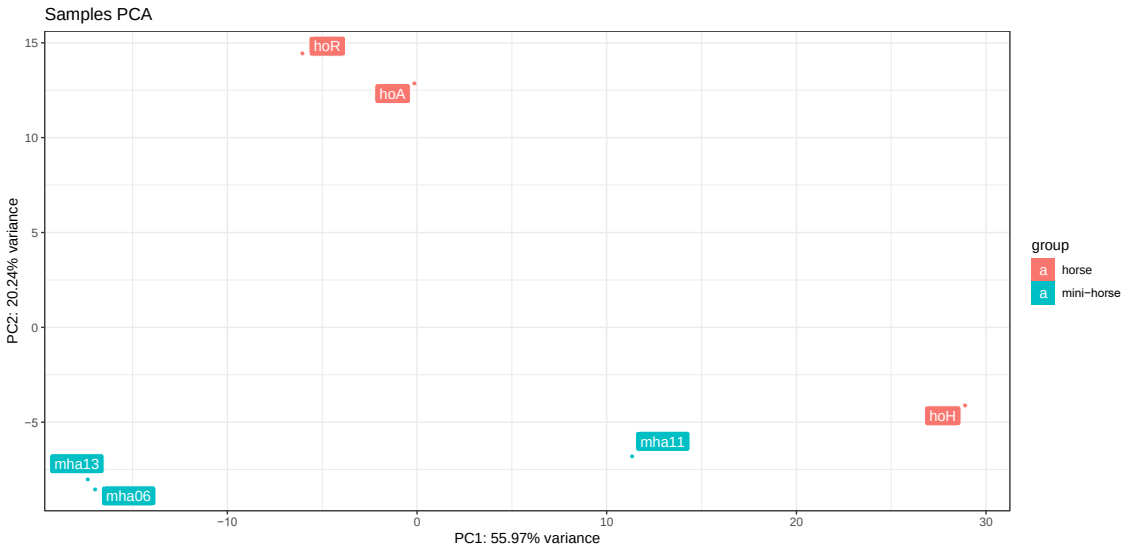

Figure 3. Transcriptome principal component analysis (PCA) indicating the presence of two distinct groups (PC1 + PC2 > 50%).

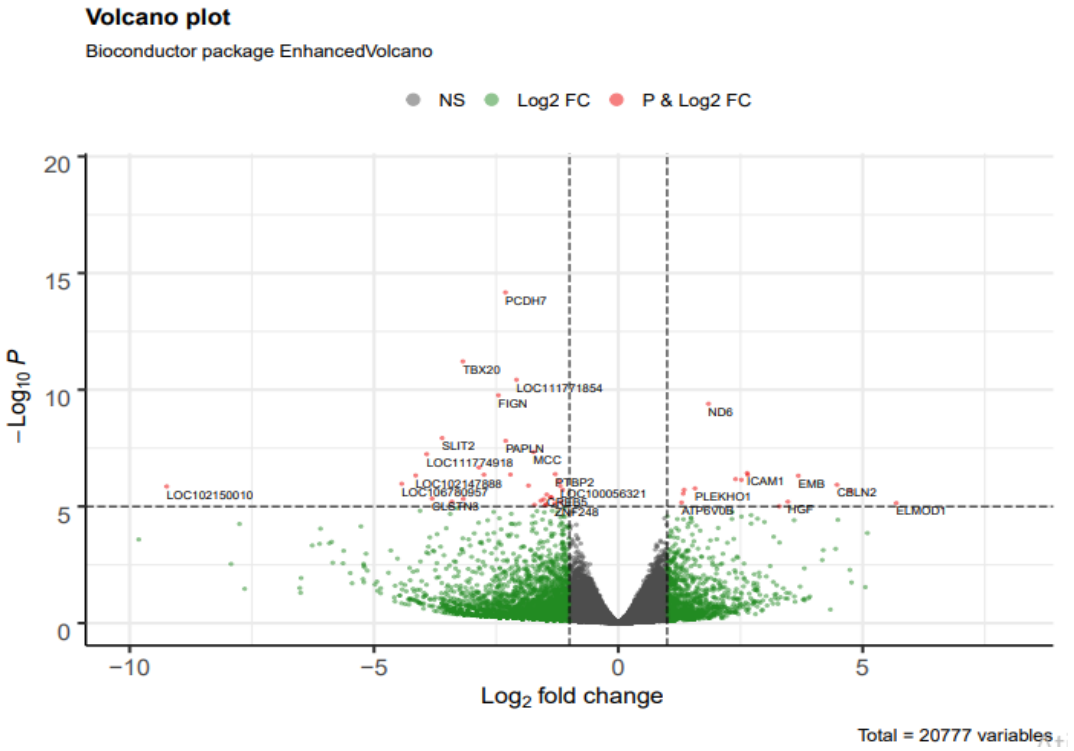

Figure 4: Volcano Plot showing on the left the most down regulated genes and on the right the most up-regulated ones.

Table 1. Most up-regulated genes, comparing mini-horses ASCs and of horses ASCs.

Gene	Description	Log2FoldChange	Pvalue	Padj
ELMOD1	ELMO Domain Containing 1; GTPase activator.	5.688487817	7.00E-06	0.002240401
EREG	Epiregulin; *growth factor activity* and epidermal growth factor receptor binding	5.100271359	0.000134219	0.014912713
DPF3	Double PHD Fingers 3; neuron-specific chromatin remodeling in neuron-specific chromatin remodeling	4.752497089	2.03E-06	0.000941942

EFHD1	EF-Hand Domain Family Member D1; calcium ion binding. Involved in a variety of cellular processes including mitosis, synaptic transmission, and cytoskeletal rearrangement.	4.492635724	3.66E-05	0.006470154
CBLN2	Cerebellin 2 Precursor. Encodes a cerebellum-specific precursor protein	4.477575142	1.14E-06	0.000695819
ANGPT1	Angiopoietin 1; Vascular development and angiogenesis.	4.449299436	0.000636935	0.040667897
SLC24A2	Solute Carrier Family 24 Member 2; Encodes a calcium/cation transport protein. This family member is a retinal cone/brain exchanger.	4.194787692	0.000727824	0.043731062
EMB	Embigin; Among its related pathways are Transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds and MAPK-Erk Pathway.	3.683743471	4.69E-07	0.000370677
KIAA1324L	KIAA1324 Like; Transmembrane protein important for cellular	3.599585628	3.77E-05	0.006582683

	response to stress.			
HGF	Hepatocyte Growth Factor*;* identical protein binding *and* serine-type endopeptidase activity.	3.471850338	6.17E-06	0.002127488
ND6	The ND6 protein is a subunit of NADH dehydrogenase (ubiquinone), which is located in the mitochondrial inner membrane	1.846355819	4.03E-10	1.08E-06
PLEKHO1	Plays a role in the regulation of the actin cytoskeleton. Also implicated in PI3K-regulated muscle differentiation and the promotion of apoptosis induced by tumor necrosis factor TNF.	1.846355819	1.70E-06	0.000876633
ICAM1	Also known as CD54 is a member of the immunoglobulin superfamily involved in intercellular adhesion	1.846355819	3.69E-07	0.000370677

Table 2. Most down-regulated genes, comparing mini-horses ASCs and of horses ASCs.

Gene	Description	Log2FoldChange	Pvalue	Padj
CADM3	Cell adhesion molecule 3; synaptic cell adhesion molecule 3	-9.814743961	0.000259525	0.02280429
RBP5	Retinol Binding Protein 5; transporter activity and retinal binding	-6.095036707	8.69E-05	0.010630784
CACNA1G	Calcium Voltage-Gated Channel. Involved in a variety of calcium-dependent processes, including muscle contraction, hormone or neurotransmitter release, gene expression, cell motility, cell division, and cell death	-5.266150133	6.97E-05	0.009368516
TBX5	T-Box Transcription Factor 5 that is essential for heart development	-4.656783328	0.000756645	0.044615527
BPIFC	BPI Fold Containing Family C; lipid binding and lipopolysaccharide binding. Associated with neutrophil granules and has antimicrobial activity.	-4.057031452	1.50E-05	0.004041358
RCAN2	Encodes a member of the regulator of calcineurin (RCAN) protein family. May play a role in	-3.885646098	0.000518156	0.035308404

	endothelial cell function and angiogenesis.			
DGLUCY	D-Glutamate Cyclase; related to Metabolism and D-Glutamine and D-glutamate metabolism.	-3.855789409	0.000205364	0.019307133
CLSTN3	Calsyntenin 3. Encoded a transmembrane calcium ion binding protein that mediate the axonal transport of certain types of vesicles.	-3.810644411	4.46E-06	0.001763523
MEOX2	Mesenchyme Homeobox 2; DNA-binding transcription factor activity and RNA polymerase II proximal promoter sequence-specific DNA binding.	-3.792132776	0.000764852	0.044902469
PDE1A	Phosphodiesterase 1ª; calmodulin binding and cGMP binding	-3.770314811	0.0008233	0.046900167
SLIT2	Glycoproteins, which are ligands for the Robo family of immunoglobulin receptors	-3.605484152	1.12E-08	2.51E-05
PCDH7	The gene encodes a protein with an extracellular domain containing 7 cadherin repeats. The gene product is an integral	-1.846355819	6.91E-15	9.28E-11

	membrane protein that function in cell-cell recognition and adhesion.			
TBX20	T-Box Transcription Factor 20 that is essential for heart development	-1.846355819	9.28E-11	3.90E-08
FIGN	ATP-dependent microtubule severing protein	-1.846355819	1.66E-10	5.59E-07

Since the therapeutically effect of the MSC is related, in great part, to their unique immunomodulatory properties and angiogenic capacity we decided to analyze these particular features in the 237 differentially expressed genes. Among the up regulated genes in the mini-horse, CD80, BMP4, LRRC32, CEBPB, TGFB1, PRNP, SMAD7 and ARG2 were related to negative regulation of T cell proliferation, negative regulation of T cell activation, negative proliferation of regulatory lymphocytes, negative regulation of mononuclear cell proliferation, negative regulation of cell-cell adhesion leukocytes, negative regulation of leukocyte proliferation, negative regulation of cell-leukocyte adhesion, negative regulation of lymphocyte activation. Figure 5 showed the functions involved in the expression of the up regulated genes, while figure 6 showed the main biological process and figure 7 the molecular function.

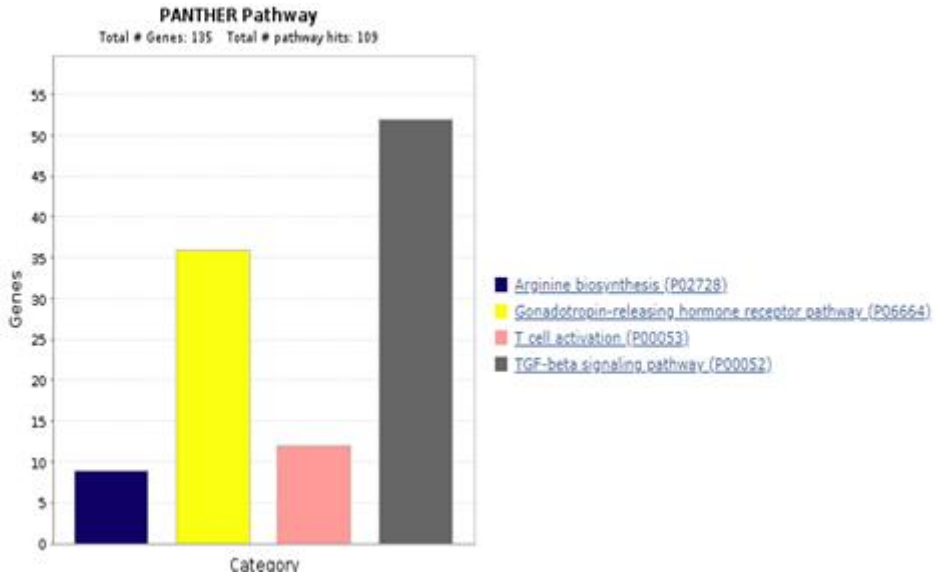

Figure 5. Biological functions involving CD80, BMP4, LRRC32, CEBPB, TGFB1, PRNP, SMAD7 and ARG2 genes, up regulated in the mini-horse.

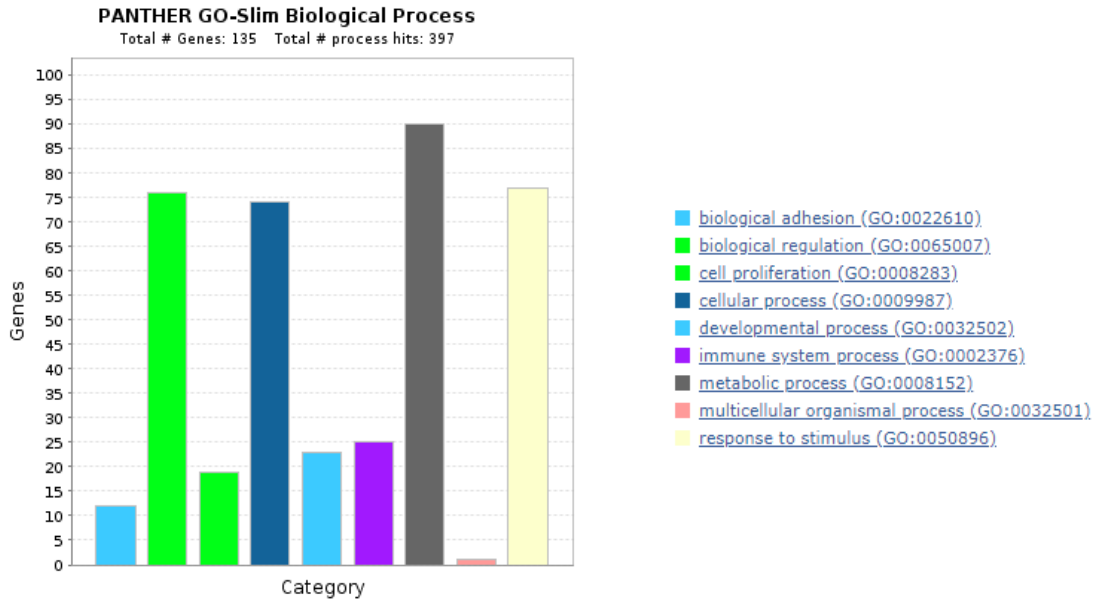

Figure 6. Biological process involving CD80, BMP4, LRRC32, CEBPB, TGFB1, PRNP, SMAD7 and ARG2 genes up regulated in the mini-horse.

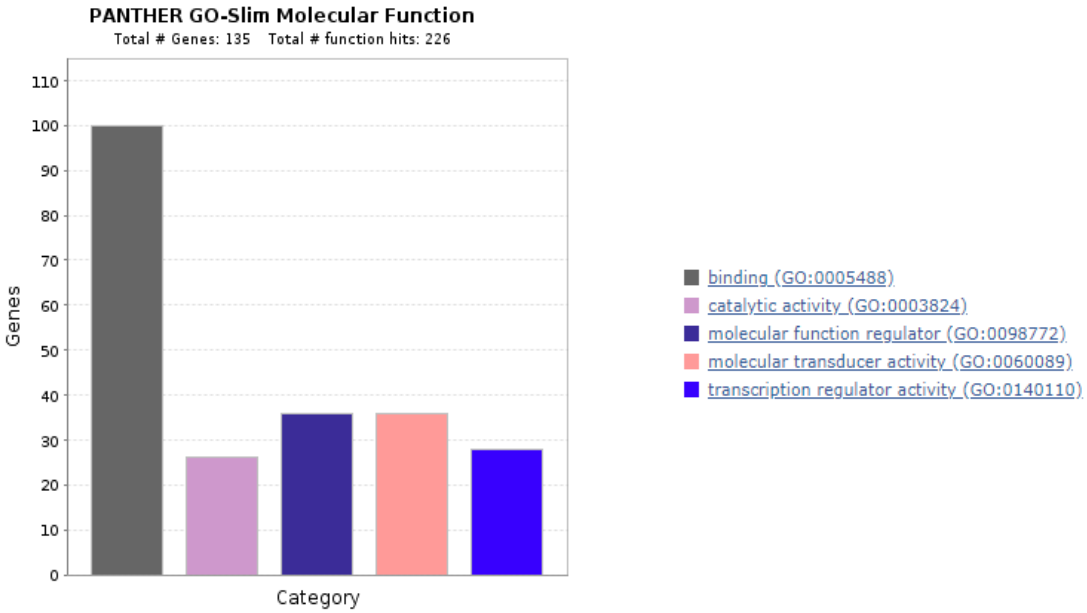

Figure 7. Molecular function involving CD80, BMP4, LRRC32, CEBPB, TGFB1, PRNP, SMAD7 and ARG2 genes up regulated in the mini-horse.

The genes correlated to angiogenesis were MEOX2, SLIT2, E2F7, TBX20, C3AR1, FGF1 and COL4A1. From this group, all presented lower expression in the mini-horse when compared to the control group (horse). These genes were also correlated to other biological functions as regulation of cell growth and cell motility Figure 8.

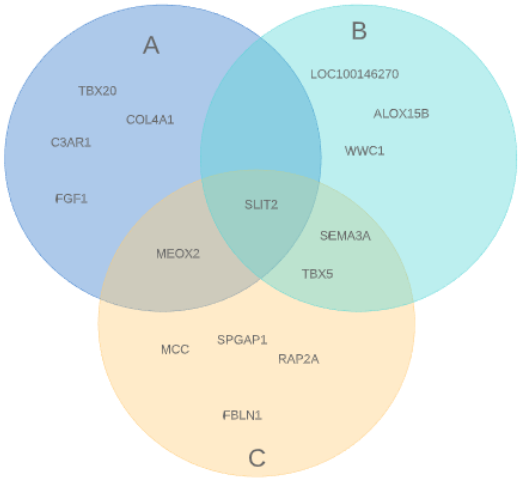

Figure 8. Venn diagram demonstrating the gene interseptions in different biological functions considering Padj value < 0.05 and Down-regulated expression, thus A represents angiogenesis; B:negative regulation of growth and C: negative regulation of cell motility.

Discussion

This study is the first to use ASCs from mini-horse, and demonstrated in first step that the use of adipose tissue is a satisfactory and easily obtainable source of mesenchymal stem cell for both mini-horses and horses, using general and local sedation anesthetic protocol and keeping the animals in season. These results corroborate with the results obtained by Bravo et al. [23] for horses. Isolation, culture, immunophenotypic characterization and the differentiation potential of the mini-horse samples did not differ from those previously obtained in our laboratory when working with horse samples [24,25]. Cultivation steps including mechanical and enzymatic digestion with collagenase and culture period in incubator at 37.5 ° C resulted in the formation of a plastic-adherent fibroblastoid cells monolayer observed from the 5th post-isolation day. Those characteristics fit the criteria attributed to mesenchymal stem cells by Dominici et al [28].

Differences in gene expression is expected in MSCs derived from different tissues, which may indicate distinct therapeutic potentials. For this reason, the

investigation of the global gene expression or transcriptome of MSC in vivo and in vitro becomes necessary in order to better understand their potential to treat diseases or regenerate tissues. In the present experiment transcriptomics analysis was used to predict similarities and differences between ASCs from mini- horses and horses.

Since the earlier studies, the transcriptome analysis of MSC showed similarities between different species [30], as well as in different populations [31]. Dessler et al. [32] noted that the transcriptome of human MSCs is relatively stable from P2 to P5 for cells expanded with the use of FBS, and the genes significantly up regulated under these conditions were related to biological processes involved in immune and inflammatory responses. These findings were also reported by Kim et al.,[33]. However, in our experiment, although all samples were cultures in the presence of FBS, the 5 more up regulated genes were related to cell proliferation and not to immunomodulation. One interesting feature observed is that, 4, from those 5 upregulated genes are preferentially expressed in the nervous tissues or related to synaptic transmission (ELMOD-1, DFP3, EFHD1 and CBLN2).

Dessler et al. [32] also observed that the expression of some genes were consistent in human-ASCs from passages 2 to 5. Samples cultures in presence of FBS present DOCK4, FIBIN, GALNT15, MMP14, RGS16, ADAMTS12, ANKRD1, BMP4 and samples cultured in presence of pHPL expressed SFRP4, THBD, PPP2R3A, FST and IL33. Moreover, some genes were common to both groups studied BCL6, CADM3, CCDC102B, ELMOD1, GUCY1A1 and GALNTS.

In our study we performed the transcriptome analysis in samples that were in P2 for mini-horses and in P5 for horses and all the genes listed above were detected in both groups. Moreover in spite the difference in culture passages, statistical differences were observed in only 1,14% of the genes. This observation agrees with Baek et al., [34] who showed that ASCs cultured in vitro presented a single transcriptome profile, regardless of the cell passage number (P1 to P5). This profile included CCR1, observed in this study in the

form of ACKR1. It was also evidenced the presence of an important aliases for the BCL6B gene, expressed by osteochondrogenic progenitor preadipocyte cells from mouse bone marrow [32] in the shape of BCL6 in this study.

Since MSC present unique immune-suppressive, modulatory, regulatory, abilities, the present experiment focused on the analysis of the expression of genes related to biological functions involved with the regenerative potential of the ASCs, like the angiogenic and the immunomodulatory properties.
Biological processes and molecular events like cell migration, angiogenesis, and tissue remodeling participate in the healing process. Angiogenesis is considered essential for the regenerative process since the formation of new blood vessels ensuring the oxygen supply, nutritional and growth factors delivery. However, in chronic wounds a decrease in angiogenesis normally delays wound healing. [35] Studies demonstrated that the use of MSCs promoted wound healing by improving angiogenesis [36], including satisfactory effects in the treatment of chronic wounds [37] The use of MSCs resulted in a paracrine effect on angiogenesis resulting in the increase levels of angiogenic factors such as angiopoietin-1 [37]. This gene AGN-1 is represented in this study for your aliases the gene ANGPT1, which has greater expression in the mini- horse group.

However, others genes associated with angiogenesis, showed higher expression in the horse ASCs. Among those the FGF1 member of the fibroblastic growth factor family, well known as an important angiogenesis inducer [38]. Also, MEOX2 is a gene related to the vascular system that plays an important role in vascular differentiation [39] and angiogenic responses [40].
The other genes like SLIT2, TBX20 and C2AR1 are indirectly involved in angiogenis, but play an important hole in cell migration and developmental mechanisms. SLIT2 play an important role in cell migration, and is involved in physiological and pathological processes [41]. TBX20 is a transcription factor essential for cardiomyocyte proliferation during development. Its absence, during pregnancy, results in embryonic death [42]. C3AR1 has been shown to bind complement anaphylatoxin C3a and was reported to be present predominantly in cortical and hippocampus neurons [43]. Its expression is also

induced in Intestinal Crypts in Response to Intestinal or Ischemia/Reperfusion [44]. The genes shared by all studied features correlated to the immunomodulatory response of ASCs were TGFB1, LRRC32, PRNP, and were significantly more expressed in the mini-horse. TGFB is significant multifunctional growth factor controlling cell proliferation and differentiation. It has an important hole on the immune system acting on T cells, B cells and myeloid cells [45]. The LRRC32 is a receptor that controls the TGFB trough the association with the latency-associated peptide (LAP), which is the regulatory chain of TGFb. By biding to LAP the LRRC32 controls TGFB1 activation on the surface of activated regulatory T-cells [45]. The PRNP gene codifies the prion protein PrP, also known as CD230. Expression of the protein is most predominant in the nervous system but occurs in many other tissues throughout the body [46]. In the Immune system it is present in hematopoietic stem cells, mature lymphoid and myeloid compartments. T cell activation is accompanied by a strong up-regulation of PrP [47].

Another gene differentially expressed (considering -0.824890864 log2FoldChange) in mini- horse group is the LRP6, an activator of beta-catenin, that causes pro-proliferation, pro-metastasis and anti-apoptosis [48]. Some polymorphisms were associated to susceptibility to the risk of cancer development [49].

The results of the present manuscript clearly demonstrated that ASCs from mini-horses and horses are very similar. However, the small differences in the transcriptome profile can potentially lead to a some different therapeutically effect. Whereas horse ASCs seems to have a more evident angiogenic and proliferative profile, the mini-horse cells seem to present a more evident immunomodulatory action. This feature, however, needs to be proved trough proteomic analysis and clinical tests to measure the efficiency of ASCs from both sub-species for use in cell therapies.

Conclusion

This study highlights the similarities and the differences in the transcriptome profile of the ASCs from mini- horses compared with horses. In summary, our results show that mini- horses ASCs have a similar transcriptome compared to horses indicating that ASCs from mini-horses can be used as an alternative to treat horse's diseases, with the vantages of presenting a higher expression of immunomodulatory genes.

Abbreviations

ASCs Adipose-derived stromal cells.
MSC Mesenchymal stromal cells.
MSC Mesenchymal stem cells.
BM Bone marrow

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CAPÍTULO III

CONSIDERAÇÕES FINAIS

Em seu conceito inicial, o presente trabalho surgiu como alternativa de potencializar a obtenção de CTMs no tratamento de enfermidades equinas, para isso viabilizando sua obtenção ao utilizar mini-horses como doadores de tecido adiposo, visto que os custos para obtenção e manutenção desses são inferiores quando comparados aos cavalos de porte regular.

Deste modo, inicialmente, esperávamos que as características gênicas expressas ao compararmos os animais fossem similares, considerando que o mini-horse é uma subespécie formada através de uma mutação de caráter recessivo no gene Agreccan.

Dados do presente estudo sugerem que apenas com exceção da angiogênese, as CTMs dos mini-horses apresentaram maiores expressões gênicas. Contudo, ainda há analises a serem realizadas como o perfil proteômico do meio condicionado produzido a partir das CTMs em cultivo, para potencializar o entendimento sobre as diferenças entre os animais estudados.