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Equine bone marrow and adipose tissue mesenchymal stem cells: cytofluorimetric characterization, in vitro differentiation and clinical application.

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Abstract

The aim of the present work was to isolate, cultivate, differentiate and conduct cellular characterization of MSCs derived from equine adipose tissue (eAT) and bone marrow (eBM). Furthermore, isolated and characterized cells were used in racehorses suffering from a superficial flexor tendon injury. eAT collection was performed at the base of the horse tail, while eBM was aspirated from iliac crest. Mononuclear cell fraction was isolated and cultured. In vitro differentiation and molecular characterization at P3 of culture were performed. No significant differences were found between DTs (Doubling Time) of all passages ($P>0.05$). DT was greater for eBM than for eAT (3.2 ± 1.5 vs 1.3 ± 0.7; $P<0.05$). Positive von Kossa and Alizarin Red staining confirmed osteogenesis. Alcian blue and Oil Red O staining illustrated chondrogenesis and adipogenesis, respectively, in both cell lines. Furthermore, isolated cells resulted positive for CD90, CD44 and CD105, while were negative for hematopoietic markers, CD14, CD45 and CD34. Although marker CD73 expresses reaction in other studies involving MSCs in different species, it did not cross-reacted with equine AT and BM mesenchymal stem cells. Using isolated cells for injured tendon therapy, no adverse reactions were observed and all inoculated horses returned to race competitions. In vitro results revealed the immunophenotypic characterization of isolated cells similar to that observed in human mesenchymal stem cells from the same sources; furthermore, in the present study, their clinical use proves the safety of equine bone marrow and adipose tissue derived MSCs and a successful outcome of the treated animals that returned to their previous level of sport activity.

Keywords: mesenchymal stem cells, bone marrow, adipose tissue, equine, characterization, tendon injuries.

1. Introduction

Isolation of mesenchymal stem cells (MSCs) has been described in several species and from different tissues, including bone marrow [1], peripheral blood [2], adult fat [3] and umbilical cord blood [4]. International Society for Cellular Therapy has established a minimal criteria for defining human MSCs [5]. They should adhere to plastic and should be able to differentiate into osteoblasts, adipocytes and chrondoblasts in vitro. Finally MSCs should express CD105, CD73 and CD90 and should not express hematopoietic markers such as CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR. Unfortunately, and in contrast to human, no such uniform characterization criteria are available for MSCs from animal origin in general, and equine origin in specific. In horses, cells from bone marrow (eBM) and adipose tissue (eAT) have been isolated and some researchers demonstrated their multilineage differentiation potential by the ability to undergo adipogenic, osteogenic and chondrogenic differentiation [6-8]. Only few authors determined immunophenotypic characterization of cells from equine adipose tissue and bone marrow [9] by flow cytometry, as request for human MSCs by ISCT [5].

Due to similarities in size, load and types of joint injuries suffered by horses and humans, U.S. Food and Drug Administration indicated the horse as the most appropriate animal model for testing clinical effects of MSCs therapies for osteoarticular injuries in human [10]. In addition, the economic and welfare costs of performance-related injuries in horses have helped to increase the interest in the use of stem cells to accelerate and improve healing [11]. Therefore, the horse can be considered at the same time an animal model for human orthopedics injuries and a patient itself [12]. Despite this premise, due to the lack of the demonstration of stem cells markers or confirmation of stemness through gene expression or differentiating capacities, in recent years, many racehorses have been treated for orthopedics injuries with cell mixture improperly called “stem cell” [13].

The aim of the present work was to isolate, cultivate, differentiate and perform flow cytometric characterization of MSCs derived from equine adipose tissue (eAT) and bone marrow (eBM), as postulated by ISCT for human cells. Furthermore, we describe the outcome of clinical cases of horses admitted to the Department of Veterinary Medical Sciences, University of Bologna, with an overstrain SDFT (Superficial Digital Flexor Tendon) lesion, after autologous eAT and eBM MSCs implantation.

2. Materials and Methods

2.1 Materials

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), plastic dishes and tubes from Sarstedt Inc.(Newton, NC, USA), unless otherwise noted.

2.2 Animals Ethics

All stages of the present study were approved by the Ethics Committee at the University of Bologna and by the Italian Ministry of Health. Before performing any manual skills on the animals, an informed consent has been signed by the owners.

2.3 Study Design

Ten racehorses, ranging in age from 2 to 9 years old, referred at the Department of Veterinary Medical Sciences, University of Bologna, due to an overstrained SDFT, were included in the present study. There was no control of age, sex or trainer for enrolled animals. The inclusion criteria were first-time tendon injuries, less than 15 days old, with an ultrasound evaluation of the cross-sectional area (CSA) >30% and, in longitudinal scans, a Fiber Alignment Score

(FAS) 2 (target path 25 to 50% parallel). It was recommended that injuries should not be recurrent but it was not possible to be certain of this for all treated horses. Animals were randomly assigned to two groups for the harvest of bone marrow (eBM; n=5) and adipose tissue (eAT; n=5). MSCs from both sources were cultured, and on the passage three (P3), they were assessed using immunophenotypic characterization by flow cytometry and evaluated for their differentiation potential into three mesenchymal lineages, as stated by ISCT for human MSCs.

2.4 Sampling and MSCs isolation

2.4.1 Bone Marrow

Bone marrow was aspirated from iliac crest of five animals (4-9 years old). Briefly, after sedation, with intravenously (IV) injection of detomidine chlorohydrate (10 μ/kg; Domosedan, Pfizer, Italy) and butorphanol tartrate (0.03 mg/kg; Nargesic ACME, Italy), the iliac crest was aseptically prepared (hair shaving and skin scrub using 10% povidone-iodine and denatured alcohol) and 2% lidocaine (Pfizer) was infiltrated into the subcutaneous tissue. BM samples were collected using 11G BM biopsy needles collected to a heparinized syringes (Eparina Vister 5000 iu/ml, Marvecspharma; ~1000 IU/10mL BM aspirate). All horses received NSAIDs (flunixin meglumine, 1.1 mg/Kg IV; Meflosyl, Pfizer) for 3 days after the procedure.

In laboratory, samples were diluted 1:1 with DPBS (Dulbecco's Phosphate Buffered Solution plus 100 iu/ml penicillin and 100 μg/ml streptomycin) and washed by centrifuging at 400 g (Heraeus Megafuge 1.0R; rotor: Heraeus # 2704), for 10 minutes. Pellet was then re-suspended in 5 ml of DMEM-TCM 199 (1:1), supplemented with 100 iu/ml penicillin, 100 μg/ml streptomycin and 10% FBS (Gibco, Invitrogen) (culture medium). The mononuclear

cell fraction was isolated by carefully loading cells onto a 70% Percoll gradient and by centrifuging at 1880 *g* for 30 minutes. Cells were collected from the interface and washed in culture medium by three centrifugation at 400 *g* for 10 minutes. After the last centrifugation, cells were re-suspended in 1 ml of culture medium and counted by hemocytometer.

2.4.2 Adipose tissue

For adipose tissue harvesting, horses were sedated as described above and the area over the dorsal gluteal muscles was aseptically prepared. Skin and subcutaneous tissues were then desensitized by local infiltration of lidocaine 2% (Pfizer) using an inverted L-block. A 10-15 cm incision was made parallel and ~15 cm abaxial to the vertebral column. Adipose tissue specimen was then harvested over the superficial gluteal fascia and placed into a 20 mL polypropylene centrifuge tube, containing sterile DPBS plus antibiotics. The skin incision was then closed with nylon suture. All horses received NSAIDs (flunixin meglumine, 1.1 mg/Kg IV; Meflosyl, Pfizer) for 3 days after the procedure.

Under a laminar flow hood, sample tissue was rinsed by repeated immersion in DPBS, weighed and minced finely (0.5 cm) using sterile scissors. Minced tissue was transferred to a 50 ml polypropylene tube, and 1 ml/1 g sample of a digestion solution (0.1 % [w/v] collagenase type I [GIBCO®, Invitrogen], dissolved in DMEM-TCM199) was added. The tissue and digestion solution were mixed thoroughly, incubated in a 37°C water bath for at least 1 hour, and mixed every 15 minutes. After incubation, collagenase was inactivated by dilution 1:1 with DPBS plus 10% (v/v) FBS. The solution obtained was filtered and undigested tissue was discarded. Nucleated cells were pelleted at 400 *g* for 10 minutes. The supernatant was discarded, pellet was re-suspended in 5 ml of culture medium and spun at 400 *g* for 10 minutes to wash cells. This operation was repeated three times. After the last

wash, cell pellet was re-suspended in 1 ml of culture medium and cell concentration was counted by hemocytometer.

2.5 Cell Doubling method

Primary cells were plated in a 25 cm^2 flask, as "Passage 0" (P0), at a density of 5 x 10^3 cell/cm^2 and incubated in a 5% CO_2 humidified atmosphere at 38.5°C. The medium was completely replaced every 3 days until the adherent cell population reached ~80% confluence. At this point, the adherent primary MSCs were passaged by digestion with 0.25% (w/v) trypsin, counted with a hemocytometer, and reseeded as P1 at 5 x 10^3 cells/cm^2. For the subsequent passages, cells were inoculated in 25 cm^2 flasks at 5 x 10^3 cells/cm^2 and allowed to multiply for 6-7 days to 90% confluence before trypsinization and successive passage. Cell-doubling time (DT), cell culture time (CT) and cell-doubling numbers (CD) were calculated from hemocytometer counts for each passage according to the following two formulae [14]:

$$CD = \ln(N_f/N_i)/\ln(2) \quad (1)$$

$$DT = CT/CD \quad (2)$$

where N_f and N_i are the final and initial number of cells, respectively.

2.6 Chondrogenic, Osteogenic and Adipogenic in vitro differentiation

During the third passage (P3) of in vitro culture, undifferentiated eBMMSCs and eATMSCs were placed in triplicate in six-well plates at density of 5x10^3 cells/cm^2 and induced towards the chondrogenic, ostegenic and adipogenic lineages, using the protocol previously described

by our research group for equine MSCs derived from foetal adnexa [15]. Briefly, after reaching 80% confluence, culture medium was removed and the differentiation media reported in Table 1 were added to the cultures. Cells in monolayer were incubated for 3 weeks. As a negative control an equal number of cells were cultured in culture medium. In both groups, the medium was completely replaced every three days. After three weeks of culture, differentiation was confirmed by appropriate staining. Briefly, to asses chondrogenic differentiation cells were fixed with 10% (v/v) formalin for 1 h at room temperature (RT), then stained with Alcian Blue solution (1% in 3% acetic acid (v/v), pH 2.5) for 15 min at RT. Alcian Blue stains acid mucosubstances and acetic mucins confirming chondrogenic differentiation cytologically. In the osteogenic assay, latter stage of osteogenesis was assessed via von Kossa and Alizarin Red staining to detect calcium or calcium salt intracellular deposits. For von Kossa staining, cells were fixed with 10% (v/v) formalin for 1 h at RT. They were then washed 5 times with distilled water then 1 ml of 5% (w/v) silver nitrate was added and cells were exposed to yellow light for 15 min. Calcium-phosphate deposits stained black. To confirm osteogenic differentiation, Alizarin Red S staining was also used. In brief, cells were rinsed with DPBS and fixed, incubating in ice-cold ethanol 70% (v/v) for 1h at RT. After three washes with distilled water, 1 ml of 2% (w/v) Alizarin Red S (pH 4.1-4.3) solution was added. The plate was incubated at RT for 30 minutes, then Alizarin Red S solution was removed and cells rinsed four times with distilled water. Calcium deposits stained red. Finally, to evaluate the baseline formation of neutral lipid-vacuoles in differentiated cells Oil Red O staining was used. Cells were fixed with 10% (v/v) formalin for 1 hour at RT. The formalin was then replaced with 2 ml of sterile water. After few minutes, water was replaced with 60% (v/v) isopropanol, then cells were covered with Oil Red O solution (0.3% in 60% isopropanol (v/v)). Five minutes later, cells were rinsed with distilled water and lipid vacuoles appeared red.

2.7 Characterization of MSCs

Cytofluorimetric analysis was performed to identify cell surface marker expression of equine MSCs. At passage 3 of culture, cells were labeled with the following monoclonal antibodies: CD105, CD45, CD90, CD44, CD34, CD14 and CD73 (all from Beckman Coulter, Fullerton, CA). They were also labeled with isotype control antibodies. Briefly, at 80% of confluence, cells were harvested using 0.25% (w/v) trypsin solution and aliquoted at a concentration of $0.5x10^6$ cells/ml. Each aliquot was fixed and permeabilized using Reagent 1 of Intraprep Kit (Beckman Coulter, Miami, FL) according to manufacturer's instructions. Cells were stained for 30 min with either conjugated-specific antibodies or istotype-matched control mouse immunoglobulin G (Table 2) at recommended concentrations. Labeled cells were washed twice in DPBS and fluorescence intensity was evaluated using a FC500 two-laser equipped cytometer (Beckman Coulter, Miami, FL). All analyses were based on control cells incubated with isotype-specific IgGs to establish the background signal. Cross reactivity of the antibodies used was screened using cultured human and horse MSCs. Furthermore, to verify cross-reactivity, control of circulating equine lymphocytes was carried out. The similarity of CD markers was also identified by comparing the amino acid sequences using Blast (Basic Local Alignment Search Tool). Results were further analysed with the CXP dedicated program.

2.8 Clinical trial

2.8.1 Cell preparation for implantation

The day of implantation, 10 ml of autologous whole venous blood was collected using a syringe pre-loaded with heparin (500 iu/ml of blood). Blood sample was centrifuged at 1500 *g* for 15 min. The obtained plasma was used to prepare implantation medium, consisting of culture medium (without FBS) plus 20% of autologous plasma.

Amplified autologous MSCs were washed three times with DPBS, trypsinized and treated as described above. After the last wash, pellet was diluted in 1 ml of implantation medium and cells were counted in a Thoma's chamber after Trypan Blue staining, to assess cell viability. The final cell concentration used was $5x10^6$ live MSCs/ml.

2.8.2 Implantation

All material used in this phase was disposable and sterile (needles, gloves, syringes). Briefly, this involved an initial ultrasonographic examination to identify the echogenicity of the core lesion and its extent in order to optimize needle placement for MSCs implantation. Horses were sedated as described above. The palmar metacarpal region was then aseptically prepared, then local subcutaneous infiltration of 2% lidocaine has been performed. After that, the cell suspension was injected into the core lesion under ultrasound guide using a 21 gauge 38-50 mm needle. After implantation, the limb was immediately bandaged to minimize subcutaneous bleeding and loss of injected cells from the tendon.

2.8.3 Rehabilitation program

After implantation, a standardized exercise program, as summarized in Table 3, was prescribed. During this period animals were not treated with any other drug (anti-inflammatory). Repeat ultrasound examinations were performed at day 0 (day of treatment), 7, 15 and 30 after treatment, to highlight possible acute side reactions to cells implantation.

Twelve months after implantation the follow up was concluded and the ability of enrolled animals to return to their previous activity was evaluated.

2.9 Statistical Analysis

To evaluate animal distribution in both groups and their homogeneity, a T student test for paired variable (Statistics for Windows, Stat Soft Inc., Tulsa, Oklahoma, USA) was performed.

Cell-doubling time, cell-doubling number and CD expression rate are expressed as mean ± standard deviation. Statistical analysis was performed using Statistics for Windows (Stat Soft Inc., Tulsa, Oklahoma, USA). Data were analysed using one-way analysis of variance (ANOVA) for multiple comparisons. Significance has been assessed for $P<0.05$.

The CSA and FAS data were reported as median and range. Kruscal Wallis test was performed to compare the value of CSA and FAS registered at day 0, 7, 15 and 30 after treatment. The analysis was performed with Medcalc, Version 12.3, and the statistically significant threshold was set up as $P< 0.05$

3. Results

3.1 Sampling and Cellular Growth

The technique used for the isolation and cultivation of MSCs derived from equine adipose (eAT) tissue and bone marrow (eBM) was proved to be safe and viable. No complications have been registered after bone marrow and adipose tissue recover.

All of the isolated cells from eAT and eBM adhered to the culture flasks on the first day of culture. Adhesion was observed within 48 hours for eBMMSCs and 24 hours for eATMSCs, and adherent cells were fibroblast-like and spindle shaped, forming a highly homogenous monolayer (Fig. 1A; Fig. 1B). During eight consecutive passages, CD of the eATMSCs was linearly increased (Fig. 2A), while eBMMSCs showed an increase of CD only until P5 (Fig. 2B). Since P0 to P8, eATMSCs showed a mean doubling time (DT) of 1.3±0.7 days/CD (range: 0.8-3.2 days). By P8, total mean CD was 37.3±4.6. The mean DT showed by eBMMSCs (P0-P5) was 3.2±1.5 days/CD (range: 0.5-5 days) and it was statistically higher than that showed by equine ATMSCs ($P<0.05$). By P5, eBMMSCs cell doubling number was 26.2±5.0. This result was not statistically different from the CD registered at P5 of equine ATMSCs ($P>0.05$). No lag phase has been observed during the in vitro culture of both cell lines: in fact no statistically significant differences in the number of CD have been registered among different culture passages ($P>0.05$).

3.2 Immunophenotypic characterization by flow cytometry

Due to no-equine specific antibodies for flow cytometry are present, in this study we used anti-human antibodies, routinely employed by Immunohaematology and Transfusion Center Equipe, Sant'Orsola Hospital, using cross-reactivity of antibodies among different species. The antibodies efficiency was verified by performing a control on circulating equine lymphocytes (data not shown). As expected, considering the results obtained with human lymphocytes, adult and hematopoietic markers used have not been expressed by these cells. Unexpected data has been registered for CD45 and CD73, that were negative also for lymphocytes (data not shown). Furthermore, we compared amino acid sequences using Blast (Basic Local Alignment Search Tool). Results are summarized in Table 4. In particular, cells

of both evaluated lines were reactive to surface markers CD90 and CD105. MSCs also demonstrated a marked reaction to CD44, a cell-surface glycoprotein having a role in MSCs migration. Typical hematopoietic cells marker (CD14) was not expressed, while there was a weak expression of CD34. Due to negative lymphocytes CD45 and CD73 expression and the lack of horse CD45 and CD73 sequence, for these markers cross-reactivity could not be confirmed, as well as its negative expression by equine BM and ATMSCs.

3.4 In vitro Differentiation

According with ISCT, we induced chondrogenic, osteogenic and adipogenic differentiation culturing each lineage for three weeks in induction media.

After three weeks of culture in chondorgenic and osteogenic induction medium, cells isolated from both tissues clearly changed their morphology from adherent monolayer of swirling spindle-shaped cells to layered cells clusters surrounded by matrix-like substance positive upon Alcian Blu (Fig. 3A; Fig. 3B) and von Kossa and Alizarin Red (Fig. 3E-H), respectively. Controls, kept in regular culture medium, showed no change in morphology and no cells stained positive (Fig. 3C-D; Fig. 3I-L). Intracytoplasmic lipid droplets were stained using Oil Red O after 21 days of in vitro culture; lipid accumulations were higher in cells cultured in adipogenic differentiation medium (Fig. 3M-N) compared to control culture (Fig. 3O-P). However, eATMSCs showed a greater adipogenic potential than eBMMSCs, characterized by a larger accumulation of lipid vacuoles (Fig. 3M-N).

3.5 Clinical outcome after eBM and eATMSC treatment

Clinical evaluation was carried both on short term (day 0 to 30 after cell injection), to highlight possible acute side reactions to cells implantation, and long term (12 months), to evaluate the ability of enrolled animals to return to their previous activity. Autologous eATMSCs and eBMMSCs implantation, re-suspended in cultured medium plus 20% of autologous plasma, did not induce any deleterious effect on the treated tissue, neither lameness, local swelling, inflammatory responses (heat or pain on palpation) or formation of abnormal tissue, detectable with ultrasound examination. No adverse reactions have been observed in any treated animal by clinical examination during the rehabilitation period.

The value of CSA registered at day 0 (inoculation) and 7, after treatment, resulted statistically higher than that observed 30 days after cells inoculation (Table 5; $P<0.05$). The same trend has been registered for FAS (Table 5; $P<0.05$). No statistically significant differences in the mean value of CSA and FAS have been registered between animals treated with eBMMSCs and eATMSCs ($P>0.05$). Twelve months after, no sign of lesion could be detected in injured tendon and fibers showed a correct alignment and a well-organized longitudinal pattern and one year after lesion occurred, all animals enrolled in the present study returned to racing.

4. Discussion

Several sources have been studied for obtaining equine MSCs [6,8,15]. However, bone marrow and adipose tissue are the most studied sources of MSCs in this species [9, 17, 18]. While bone marrow aspiration from sternum is considered as a quick and innocuous method of harvest, there have been case reports of accidental fatal thoracic and cardiac puncture [19] and nonfatal pneumopericardium [20] during bone marrow aspiration from the sternum. Although these cases are rare, they probably resulted from poor appreciation of local topographical anatomy at the aspiration site and hence a failure to identify the appropriate site and depth of needle placement [21]. Some Authors reported a site injuries also after adipose

tissue collection [22]. In the present study, no side effect have been observed after adipose tissue and bone marrow harvest, demonstrating that the surgical collection of adipose tissue from the base of the tail and the aspiration of bone marrow from iliac crest are viable and safe for animals. Obtained results verified the adherence of eATMSCs and eBMMSCs in culture in <48 hours, a fact in agreement with reports in previously published data concerning the characteristics of these cells to adhere to plastic when maintained in culture conditions [8, 17]. Different from other Authors [14], in the present study, cells isolated from bone marrow and adipose tissue did not show a lag phase during their in vitro culture. However, while previous researches did not report a significantly different proliferation rate between eBMMSCs and eATMSCs [17] or show a higher doubling time for eATMSCs [8], in the present study cells isolated from equine adipose tissue are characterized by a lower DT compared with cells isolated from equine bone marrow cultured under the same in vitro conditions. Furthermore, different from eBMMSCs, eATMSCs can be grown for longer time in vitro. These characteristics could be very important for using these cells for autologous therapy. Right now, autologous therapy with MSCs is widely used because, as shown by the present study, it does not result in any significant deleterious effects at the time of implantation or later, and shows anti-inflammatory and immunosuppressive effects [23]. However, treatment with autologous MSCs has limitations, such as in acute injuries, because expansion of MSCs by culturing takes different days. Cellular growing data registered by us, similar healing time and no side effects registered in both groups, would make adipose tissue an advantageous source for cellular therapy. Moreover, since no side effects have been observed using these cells for allogenic therapy [unpublished data, 24], it would make adipose tissue also an important MSCs resource for allogenic bank.

Minimum criteria for the characterization of human MSCs, postulated by ISCT [5], consider not only the ability of cells to adhere to the plastic when maintained in vitro and their

proliferation rate. In fact, an important feature of MSCs is the expression of markers CD105, CD73, and CD90 and the lack for markers CD45, CD34, CD14, the human leukocyte antigen-DR surface molecules. Furthermore, as postulated by Dominici et al [5], human MSCs should present the capacity to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro. In the present study, eAT and eBM were used as sources for obtaining equine MSCs, and the MSCs' expression of surface markers and their differentiation potential into osteogenic, adipogenic and chondrogenic lineages were evaluated, as stated for human MSCs. All these determinations were performed at the third passage of in vitro culture because the cells reached homogeneous culture at this point, as demonstrated by previous studies [15, 18]. In our study, differentiation into osteoblasts was confirmed by staining calcium deposits with Alizarin red and Von Kossa. The osteogenic differentiation in equine MSCs was faster than in other species, including human [25], porcine [26] (Zou *et al*. 2008) and bovine [27] (Bosnakovski *et al*. 2005) and similar to horses [14]. We cultured isolated cells in adipogenic medium supplemented with 15% of rabbit serum, as recently reported also by our research group for equine MSCs isolated by foetal adenexa [15]. In fact, it was found that rabbit serum enhanced adipogenesis *in vitro* for human [28], rat and mouse [29] MSCs. Rabbit serum has a high content of free fatty acids, which are putative ligands of PPAR*y* and may thus enhance adipogenesis. Recently, Ranera et al [18], comparing different induction media for adipogenic differentiation of equine MSCs, found that only the medium supplemented with 15% rabbit serum was able to induce adipogenic differentiation. Other authors did not find necessary the addition of rabbit serum to achieve any reliable adipogenesis [30]. The intracellular accumulation of red-stained lipid droplets on Day 21 of culture was indicative of adipogenic differentiation. However, cytoplasmic droplets were already visible within the first few days of culture. This characteristic was in agreement with findings observed by other authors [8,

30]. Different from that observed in another study [18], in the present research equine MSCs did not display an adipogenic potential lower than other species.

As a final step in the differentiation process, we demonstrated that equine MSCs had tri-lineage potential since cells were able to differentiate into chondrocytes. Cells isolated from eAT showed a higher differentiation potency as demonstrated by a greater accumulation of glycosaminoglycans, calcium salt and lipid droplet, comparing with cells isolated from eBM and cultured under the same differentiation condition. These results are not in agreement with those that proved the lack of significant differences between the two lines [31] or reported that osteogenic and chondrogenic differentiation can be better in eBMMSCs [32,33]. Further studies could be conducted to assess the differentiation potential of eAT and eBMMSCs in other lineages of therapeutic interest, such as myocytes, and further investigation are needed using quantitative PCR to confirm a distinct differentiation potential between adipose and bone marrow derived cells.

Different from previous studies [18, 31], during the present experiment, the immunophenotypic characterization of the surface of the MSCs used was conducted, by flow cytometry, with the same markers considered in humans, excepted for equine leukocyte antigen-DR surface molecules because of the lack of equine specific monoclonal antibodies available and evidence that certain markers from other species do not cross-react with the equine species [34]. To provide evidence for inter-species cross-reactivity, the similarity of CD markers between human and equine, was identified comparing the amino acid sequence, as suggested by de Mattos Carvalho et al. [35], and we used equine circulating lymphocytes as control [15]. The immunophenotypic investigation was conducted only by flow cytometry, and PCR was not employed. In fact, although this technique shows the mRNA expression of different markers, this expression is not always correlated with the presence of protein, therefore with stemness. However, mRNA expression detection by PCR may possibly

complement the results obtained. In agreement with our previous report in horses [15], eBM and eAT, cultured under the same conditions, showed high positivity for CD90 and CD44. CD90, called Thy-1, is an antigen present in established culture of equine MSC. The expression profile of these markers in equine BM- and AT-MSCs at passage 3, was in accordance with the immunophenotype reported for human MSCs by ISCT [5]. The CD44 antigen is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration. Data observed in the present study confirm those previously reported, by our team, for MSCs isolated from equine foetal adnexa [15]. Relatively low CD105 expression relative to CD90 and CD44 by both MSCs lines has been previously reported in equine by Xie et al [36]. CD105 (endoglin) is a high affinity co-receptor for transforming growth factor (TGF)-β1 and TGF-β3 [37]. Although CD105 is generally considered an important marker for MSCs [5] several reports showed that its expression vary depending upon MSC source, culture time in vitro and differentiation state [38,39]. In human and mouse the existence of a heterogeneous cell population CD105 positive and CD105 negative have been recently demonstrated [40, 41]. Furthermore, since CD105 is a component of the TGF-β receptor, its presence or absence on the MSCs must have an effect on their response to TGF-β. In particular, MSCs constitutively secrete TGF-β1 in culture and the fetal bovine serum contains high levels of latent TGF-β1 [40, 42], so the expression of this protein could be related to the culture medium composition, and in particular to the presence of serum, as observed recently in human by Mark et al [43]. A lack of reactivity with haematopoietic markers CD14, which cross-reaction was confirmed by lymphocytes investigation, indicates that isolated cells are negative for haematopoietic progenitors. On the other hand isolated cells showed a weak expression of CD34 in both eAT and eBMMSCs, despite in a higher percentage compared with the findings of Ranera et al [9] but without statistically significant differences between the two lineages. Another study on equine MSCs from adipose tissue and bone marrow stated

its lack in these lineages [31]. Because the immunoreactivity for CD34 in human AT-MSCs declines with passages [44,45], further analysis is necessary to confirm that the loss of CD34 in equine MSCs is similar to that in human cells. Furthermore, the lack of reactivity of equine cells and lymphocytes with the haematopoietic markers CD45 and MSC with marker CD73 probably indicates that the human-directed reagents do not cross-react with their corresponding equine epitopes. These findings need further investigation to assess if, in particularly, the lack of CD73 expression is due to the lack of cross-reactivity or is a species-specific feature, due to the same findings in previous studies conducted both on eATMSCs and on foetal MSCs [15, 46]. However, taken together, the results obtained in the present study support an MSC phenotype from both tissue sources used in this investigation.

No significant increase in lesion cross-sectional area or pain sensitivity occurred after the implantation of adipose and bone marrow derived MSCs, which is in agreement with the results reported by Fortier and Smith [47], who indicated that the implantation of bone marrow-derived MSCs did not provoke worsening of the lesion or even tendon reaction, with no increase in tendon area in ultrasonographic imaging. The dose of progenitor cells used in this study ($5x10^6$ cells) is lower than that used by other Authors [48]. At present, there are no published studies evaluating the optimal number of MSCs that should be used in the treatment of tendinitis, though one recent report suggests that murine MSCs are potentially cytotoxic when injected in high concentrations directly into tumor tissue (melanoma), liberating several angiogenesis inhibitor agents that induce apoptosis and annul tumor growth, a process that would be of enormous potential in cancer therapy [49]. Whether the administration of high concentrations of MSCs in tendon injuries stimulates the release of angiogenesis inhibitors remains unknown, though this occurs, it could result in the inhibition of tendon healing, which is not desirable. In the present study, the use of eAT and eBMMSCs proved to be safe with the absence of neoplastic tissue formation at the lesion site where the implantation was

performed during the experiment. Analysis of the results of the ultrasonographic evaluation of the tendons is in agreement with previously published reports [48]. Furthermore, different from Barreira et al. [50], no ultrasonographic differences in the mean values of the percentage of ruptured collagen fibers in a cross-sectional view have been observed after the administration of MSCs. In our experiment, all ultrasonographic imaging was obtained by the same operator using the same ultrasound equipment, to avoid variation due to different operators and the use of different equipment. This precaution is extremely important because it was demonstrated that significant interoperator variability can occur when measuring the area of the same tendon [51]. Despite the positive results obtained in the present study we are aware that it has some limitations. In the present study owned horses with overstrain SDF injuries have been enrolled, no control group was included neither an animal treated as clinical case has been subjected to histological examination, differently from studies performed in experimental animals [48, 50].

Different Authors, for treating induced tendon lesions, performed the implantation of mononucleated cells, derived from adipose and bone marrow tissue, 48 hours after harvest, and they called the mix of cells “stem cells”, though MSCs are present in small quantities. In our study, autologous adipose tissue and bone marrow-derived MSCs isolated, expanded in vitro and characterized have been used. Our choice involves greater cost, it is more laborious, and obviously the application requires a delay in the therapy needed for cell expansion in vitro. However, it has an advantage in that the procedure permits isolation and expansion of the number of MSCs, thereby avoiding the administration of a heterogenous cell population that can disturb the process of tendon repair [52].

5. Conclusion

The panel of surface antigens tested by flow cytometry in the present study revealed a similar phenotypic profile between horse and human MSCs, although specific differences in some

surface antigens were noticed. A similar cell surface profile was also observed between eBMMSCs and eATMSCs. This findings are important for characterizing these cells before using them for cellular-based therapies in equine medicine. However, many questions still remain, and further investigation will be necessary to clarify the mechanisms and functions of stem cell epitopes, such as the effect of marker expression variation on the pluripotency of MSCs or the study of their expression by cells from different passages. Furthermore, though further investigation are needed using a higher number of animals, our clinical data confirm that eAT and eBMMSCs could be used in clinical trials involving both autologous and allogeneic therapy in horses. Under the experimental conditions of this study, the eATMSCs showed higher in vitro differentiation and cell growth. These findings suggest that eAT may be preferable for cell banking purposes.

6. Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the reported research.

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690 **Table 1.** Media used for inducing adipogenic, osteogenic and chondrogenic differentiation of

691 cells isolated from eAT and eBM.

Differentiation	Medium	Serum %	Supplements
Control	DMEM-TCM199	10% FBS	100 IU/ml penicillin, 100 μg/ml streptomycin
Adipogenic	DMEM-TCM199	15% Rabbit Serum	100 IU/ml penicillin, 100 μg/ml streptomycin, 1 μM dexamethasone (for 6 days), 0.5 mM isobutyl-methylxanthine (for 3 days), 10 mM insulin, 0.2 mM indomethacin
Osteogenic	DMEM-TCM199	10% FBS	100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM β- glycerophosphate, 0.1 μM dexamethasone, 50 μM ascorbic acid
Condrogenic	DMEM-TCM199	1% FBS	100 IU/ml penicillin, 100 μg/ml streptomycin, 6.25 μg/ml insulin, 50 mM ascorbic acid, 0.1 μM dexamethasone, 10 ng/ml human Tranforming Growth Factor-β1

692

693 **Table 2.** Primary antibodies and Isotypes used for flow cytometry.

Markers	Primary antibody	Ig
CD44FITC	Mouse monoclonal	IgG1
CD90PC5	Mouse monoclonal	IgG1
CD105PE	Mouse monoclonal	IgG2a
CD73PE	Mouse monoclonal	IgG1
CD14PC5	Mouse monoclonal	IgG2a
CD45APC	Mouse monoclonal	IgG1
Isotype		
Isotype PC5	Mouse monoclonal	IgG2a
Isotype FITC	Mouse monoclonal	IgG1
Isotype PE	Mouse monoclonal	IgG1
Isotype APC	Mouse monoclonal	IgG1

694 Ig, immunoglobin

695

696

Table 3. Rehabilitation program after cell inoculation.

Week after cell inoculation	Exercise program
1	Box rest
2-4	Hand walk 10 min twice/day
5-9	Hand walk 20 min twice /day
10-16	Hand walk 30-40 min twice /day
17-25	Hand walk 40 min twice /day and trot 5-30 min/day
26-52	Gradual increase of exercise level

Table 4. Flow cytometry analysis of eATMSCs and eBMMSCs at Passage 3 of in vitro culture. Summarizing table.

Tissue	CD90	CD105	CD73	CD44	CD14	CD34	CD45
eAT	69.5±8.4	70.5±1.8	0.2±0.3	91.9±8.9	0.6±0.3	5.8±4.8	3.0±4.2
eBM	66.1±28.4	62.5±10.6	2.7±2.1	97.6±1.3	1.1±0.3	7.7±9.2	9.0±11.5

704 **Table 5.** Cross Sectional Area (CSA) and Fibers Alignment Score (FAS): median and range
705 obtained by ultrasound examination. Day 0: day of inoculation. a vs b P<0.05;*P<0.01.

706

Parameters	Median (Range)		Median (Range)	
	Day 0	Day7	Day 15	Day 30
CSA %	30[a] (20-50)	30[a] (20-50)	30[a] (10-50)	20*[b] (10-30)
FAS	2[a] (1-3)	2[a] (1-3)	1[b] (1-2)	1*[a] (1-2)

Figure Legends

Figure 1. Monolayer of rapidly expanding adherent spindle-shaped fibroblastoid cells compatible with undifferentiated mesenchymal stem cell. Adipose Tissue (A), Bone Marrow (B). Magnification x 10.

Figure 2. Cell doubling time and number of cultured primary and passaged mesenchymal stem cells. All values reflect the mean ± standard deviation. A-B: Adipose Tissue. C-D: Bone Marrow.

Figure 3. Overlay histograms of cytometry analysis. In black isotypic controls are represented. Empty histograms represent the analysis with mAbs on mesenchymal cell culture.

Figure 4. In vitro differentiation studies. (A) Chondrogenic induction in eATMSCs over three weeks: Alcian Blue staining of glycosaminoglycans in cartilage matrix. (B) Chondrogenic induction in eBMMSCs over three weeks: Alcian Blue staining of glycosaminoglycans in cartilage matrix. (C)-(D) Chondrogenic control: eAT and eBM MSCs cultured in regular medium for 21 days maintained normal morphology and stained negative for Alcian Blue. (E) Osteogenic induction in eATMSCs over three weeks: von Kossa staining of extensive extracellular calcium deposition. (F) Osteogenic induction in eBMMSCs over three weeks: von Kossa staining of extensive extracellular calcium deposition. (G) Osteogenic induction in eATMSCs over three weeks: Alizarin Red staining of extensive extracellular calcium deposition. (H) Osteogenic induction in eBMMSCs over three weeks: Alizarin Red staining of extensive extracellular calcium deposition. (I)-(J) Osteogenic control: eAT and eBM MSCs cultured in standard medium for 21 days maintained normal morphology and stained negative

for von Kossa staining. (K)-(L): Osteogenic control: eAT and eBM MSCs cultured in regular medium, after 21 days presented normal morphology and stained negative for Alizarin Red staining. (M) Adipogenic induction in eATMSCs over three weeks: Oil red O staining of extensive intracellular lipid droplet accumulation. (N) Adipogenic induction in eBMMSCs over three weeks: Oil red O staining of extensive intracellular lipid droplet accumulation. (O)-(P) Adipogenic control: eATMSCs and eBMMSCs, after 21 days of culture in standard medium presented normal morphology and stained negative for Oil red O staining.