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Eleonora Iacono, Barbara Merlo, Noemi Romagnoli, Barbara Rossi, Francesca Ricci, Alessandro Spadari. Equine Bone Marrow and Adipose Tissue Mesenchymal Stem Cells: Cytofluorimetric Characterization, In Vitro Differentiation, and Clinical Application. Journal of Equine Veterinary Science, 35 (2015) 130–140.

The final published version is available online at: https://doi.org/10.1016/j.jevs.2014.12.010

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

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38

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.

65 **1. Introduction**

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

80 Due to similarities in size, load and types of joint injuries suffered by horses and humans,
81 U.S. Food and Drug Administration indicated the horse as the most appropriate animal model
82 for testing clinical effects of MSCs therapies for osteoarticular injuries in human [10]. In
83 addition, the economic and welfare costs of performance-related injuries in horses have
84 helped to increase the interest in the use of stem cells to accelerate and improve healing [11].
85 Therefore, the horse can be considered at the same time an animal model for human
86 orthopedics injuries and a patient itself [12]. Despite this premise, due to the lack of the
87 demonstration of stem cells markers or confirmation of stemness through gene expression or
88 differentiating capacities, in recent years, many racehorses have been treated for orthopedics
89 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

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

122

123 ***2.4 Sampling and MSCs isolation***

124

125 *2.4.1 Bone Marrow*

126 Bone marrow was aspirated from iliac crest of five animals (4-9 years old). Briefly, after
127 sedation, with intravenously (IV) injection of detomidine chlorohydrate (10 µ/kg;
128 Domosedan, Pfizer, Italy) and butorphanol tartrate (0.03 mg/kg; Nargesic ACME, Italy), the
129 iliac crest was aseptically prepared (hair shaving and skin scrub using 10% povidone-iodine
130 and denatured alcohol) and 2% lidocaine (Pfizer) was infiltrated into the subcutaneous tissue.
131 BM samples were collected using 11G BM biopsy needles collected to a heparinized syringes
132 (Eparina Vister 5000 iu/ml, Marvecspharma; ~1000 IU/10mL BM aspirate). All horses
133 received NSAIDs (flunixin meglumine, 1.1 mg/Kg IV; Meflosyl, Pfizer) for 3 days after the
134 procedure.
135 In laboratory, samples were diluted 1:1 with DPBS (Dulbecco's Phosphate Buffered Solution
136 plus 100 iu/ml penicillin and 100 μg/ml streptomycin) and washed by centrifuging at 400 *g*
137 (Heraeus Megafuge 1.0R; rotor: Heraeus # 2704), for 10 minutes. Pellet was then re-
138 suspended in 5 ml of DMEM-TCM 199 (1:1), supplemented with 100 iu/ml penicillin, 100
139 μg/ml streptomycin and 10% FBS (Gibco, Invitrogen) (culture medium). The mononuclear

140 cell fraction was isolated by carefully loading cells onto a 70% Percoll gradient and by
141 centrifuging at 1880 *g* for 30 minutes. Cells were collected from the interface and washed in
142 culture medium by three centrifugation at 400 *g* for 10 minutes. After the last centrifugation,
143 cells were re-suspended in 1 ml of culture medium and counted by hemocytometer.
144
145 *2.4.2 Adipose tissue*
146 For adipose tissue harvesting, horses were sedated as described above and the area over the
147 dorsal gluteal muscles was aseptically prepared. Skin and subcutaneous tissues were then
148 desensitized by local infiltration of lidocaine 2% (Pfizer) using an inverted L-block. A 10-15
149 cm incision was made parallel and ~15 cm abaxial to the vertebral column. Adipose tissue
150 specimen was then harvested over the superficial gluteal fascia and placed into a 20 mL
151 polypropylene centrifuge tube, containing sterile DPBS plus antibiotics. The skin incision was
152 then closed with nylon suture. All horses received NSAIDs (flunixin meglumine, 1.1 mg/Kg
153 IV; Meflosyl, Pfizer) for 3 days after the procedure.
154 Under a laminar flow hood, sample tissue was rinsed by repeated immersion in DPBS,
155 weighed and minced finely (0.5 cm) using sterile scissors. Minced tissue was transferred to a
156 50 ml polypropylene tube, and 1 ml/1 g sample of a digestion solution (0.1 % [w/v]
157 collagenase type I [GIBCO®, Invitrogen], dissolved in DMEM-TCM199) was added. The
158 tissue and digestion solution were mixed thoroughly, incubated in a 37°C water bath for at
159 least 1 hour, and mixed every 15 minutes. After incubation, collagenase was inactivated by
160 dilution 1:1 with DPBS plus 10% (v/v) FBS. The solution obtained was filtered and
161 undigested tissue was discarded. Nucleated cells were pelleted at 400 *g* for 10 minutes. The
162 supernatant was discarded, pellet was re-suspended in 5 ml of culture medium and spun at
163 400 *g* for 10 minutes to wash cells. This operation was repeated three times. After the last

164 wash, cell pellet was re-suspended in 1 ml of culture medium and cell concentration was
165 counted by hemocytometer.
166
167 ***2.5 Cell Doubling method***
168
169 Primary cells were plated in a 25 cm^2 flask, as "Passage 0" (P0), at a density of 5 x 10^3
170 $cell/cm^2$ and incubated in a 5% CO_2 humidified atmosphere at 38.5°C. The medium was
171 completely replaced every 3 days until the adherent cell population reached ~80% confluence.
172 At this point, the adherent primary MSCs were passaged by digestion with 0.25% (w/v)
173 trypsin, counted with a hemocytometer, and reseeded as P1 at 5 x 10^3 $cells/cm^2$. For the
174 subsequent passages, cells were inoculated in 25 cm^2 flasks at 5 x 10^3 $cells/cm^2$ and allowed
175 to multiply for 6-7 days to 90% confluence before trypsinization and successive passage.
176 Cell-doubling time (DT), cell culture time (CT) and cell-doubling numbers (CD) were
177 calculated from hemocytometer counts for each passage according to the following two
178 formulae [14]:

179 $$CD= \ln(N_f/N_i)/\ln(2) \qquad (1)$$

180

181 $$DT= CT/CD \qquad (2)$$

182 where N_f and N_i are the final and initial number of cells, respectively.
183
184 ***2.6 Chondrogenic, Osteogenic and Adipogenic in vitro differentiation***
185
186 During the third passage (P3) of in vitro culture, undifferentiated eBMMSCs and eATMSCs
187 were placed in triplicate in six-well plates at density of $5x10^3$ $cells/cm^2$ and induced towards
188 the chondrogenic, ostegenic and adipogenic lineages, using the protocol previously described

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

214

215 ***2.7 Characterization of MSCs***

216

217 Cytofluorimetric analysis was performed to identify cell surface marker expression of equine
218 MSCs. At passage 3 of culture, cells were labeled with the following monoclonal antibodies:
219 CD105, CD45, CD90, CD44, CD34, CD14 and CD73 (all from Beckman Coulter, Fullerton,
220 CA). They were also labeled with isotype control antibodies. Briefly, at 80% of confluence,
221 cells were harvested using 0.25% (w/v) trypsin solution and aliquoted at a concentration of
222 $0.5x10^6$ cells/ml. Each aliquot was fixed and permeabilized using Reagent 1 of Intraprep Kit
223 (Beckman Coulter, Miami, FL) according to manufacturer's instructions. Cells were stained
224 for 30 min with either conjugated-specific antibodies or istotype-matched control mouse
225 immunoglobulin G (Table 2) at recommended concentrations. Labeled cells were washed
226 twice in DPBS and fluorescence intensity was evaluated using a FC500 two-laser equipped
227 cytometer (Beckman Coulter, Miami, FL). All analyses were based on control cells incubated
228 with isotype-specific IgGs to establish the background signal. Cross reactivity of the
229 antibodies used was screened using cultured human and horse MSCs. Furthermore, to verify
230 cross-reactivity, control of circulating equine lymphocytes was carried out. The similarity of
231 CD markers was also identified by comparing the amino acid sequences using Blast (Basic
232 Local Alignment Search Tool). Results were further analysed with the CXP dedicated
233 program.

234

235 ***2.8 Clinical trial***

236

237 *2.8.1 Cell preparation for implantation*

238 The day of implantation, 10 ml of autologous whole venous blood was collected using a
239 syringe pre-loaded with heparin (500 iu/ml of blood). Blood sample was centrifuged at 1500 *g*
240 for 15 min. The obtained plasma was used to prepare implantation medium, consisting of
241 culture medium (without FBS) plus 20% of autologous plasma.
242 Amplified autologous MSCs were washed three times with DPBS, trypsinized and treated as
243 described above. After the last wash, pellet was diluted in 1 ml of implantation medium and
244 cells were counted in a Thoma's chamber after Trypan Blue staining, to assess cell viability.
245 The final cell concentration used was $5x10^6$ live MSCs/ml.
246
247 *2.8.2 Implantation*
248 All material used in this phase was disposable and sterile (needles, gloves, syringes). Briefly,
249 this involved an initial ultrasonographic examination to identify the echogenicity of the core
250 lesion and its extent in order to optimize needle placement for MSCs implantation. Horses
251 were sedated as described above. The palmar metacarpal region was then aseptically
252 prepared, then local subcutaneous infiltration of 2% lidocaine has been performed. After that,
253 the cell suspension was injected into the core lesion under ultrasound guide using a 21 gauge
254 38-50 mm needle. After implantation, the limb was immediately bandaged to minimize
255 subcutaneous bleeding and loss of injected cells from the tendon.
256
257 *2.8.3 Rehabilitation program*
258 After implantation, a standardized exercise program, as summarized in Table 3, was
259 prescribed. During this period animals were not treated with any other drug (anti-
260 inflammatory). Repeat ultrasound examinations were performed at day 0 (day of treatment),
261 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.

286 All of the isolated cells from eAT and eBM adhered to the culture flasks on the first day of
287 culture. Adhesion was observed within 48 hours for eBMMSCs and 24 hours for eATMSCs,
288 and adherent cells were fibroblast-like and spindle shaped, forming a highly homogenous
289 monolayer (Fig. 1A; Fig. 1B). During eight consecutive passages, CD of the eATMSCs was
290 linearly increased (Fig. 2A), while eBMMSCs showed an increase of CD only until P5 (Fig.
291 2B). Since P0 to P8, eATMSCs showed a mean doubling time (DT) of 1.3±0.7 days/CD
292 (range: 0.8-3.2 days). By P8, total mean CD was 37.3±4.6. The mean DT showed by
293 eBMMSCs (P0-P5) was 3.2±1.5 days/CD (range: 0.5-5 days) and it was statistically higher
294 than that showed by equine ATMSCs ($P<0.05$). By P5, eBMMSCs cell doubling number was
295 26.2±5.0. This result was not statistically different from the CD registered at P5 of equine
296 ATMSCs ($P>0.05$). No lag phase has been observed during the in vitro culture of both cell
297 lines: in fact no statistically significant differences in the number of CD have been registered
298 among different culture passages ($P>0.05$).
299
300 ***3.2 Immunophenotypic characterization by flow cytometry***
301
302 Due to no-equine specific antibodies for flow cytometry are present, in this study we used
303 anti-human antibodies, routinely employed by Immunohaematology and Transfusion Center
304 Equipe, Sant'Orsola Hospital, using cross-reactivity of antibodies among different species.
305 The antibodies efficiency was verified by performing a control on circulating equine
306 lymphocytes (data not shown). As expected, considering the results obtained with human
307 lymphocytes, adult and hematopoietic markers used have not been expressed by these cells.
308 Unexpected data has been registered for CD45 and CD73, that were negative also for
309 lymphocytes (data not shown). Furthermore, we compared amino acid sequences using Blast
310 (Basic Local Alignment Search Tool). Results are summarized in Table 4. In particular, cells

311 of both evaluated lines were reactive to surface markers CD90 and CD105. MSCs also
312 demonstrated a marked reaction to CD44, a cell-surface glycoprotein having a role in MSCs
313 migration. Typical hematopoietic cells marker (CD14) was not expressed, while there was a
314 weak expression of CD34. Due to negative lymphocytes CD45 and CD73 expression and the
315 lack of horse CD45 and CD73 sequence, for these markers cross-reactivity could not be
316 confirmed, as well as its negative expression by equine BM and ATMSCs.
317
318 ***3.4 In vitro Differentiation***
319
320 According with ISCT, we induced chondrogenic, osteogenic and adipogenic differentiation
321 culturing each lineage for three weeks in induction media.
322 After three weeks of culture in chondorgenic and osteogenic induction medium, cells isolated
323 from both tissues clearly changed their morphology from adherent monolayer of swirling
324 spindle-shaped cells to layered cells clusters surrounded by matrix-like substance positive
325 upon Alcian Blu (Fig. 3A; Fig. 3B) and von Kossa and Alizarin Red (Fig. 3E-H),
326 respectively. Controls, kept in regular culture medium, showed no change in morphology and
327 no cells stained positive (Fig. 3C-D; Fig. 3I-L). Intracytoplasmic lipid droplets were stained
328 using Oil Red O after 21 days of in vitro culture; lipid accumulations were higher in cells
329 cultured in adipogenic differentiation medium (Fig. 3M-N) compared to control culture (Fig.
330 3O-P). However, eATMSCs showed a greater adipogenic potential than eBMMSCs,
331 characterized by a larger accumulation of lipid vacuoles (Fig. 3M-N).
332
333 ***3.5 Clinical outcome after eBM and eATMSC treatment***
334

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

360 tissue collection [22]. In the present study, no side effect have been observed after adipose
361 tissue and bone marrow harvest, demonstrating that the surgical collection of adipose tissue
362 from the base of the tail and the aspiration of bone marrow from iliac crest are viable and safe
363 for animals. Obtained results verified the adherence of eATMSCs and eBMMSCs in culture
364 in <48 hours, a fact in agreement with reports in previously published data concerning the
365 characteristics of these cells to adhere to plastic when maintained in culture conditions [8, 17].
366 Different from other Authors [14], in the present study, cells isolated from bone marrow and
367 adipose tissue did not show a lag phase during their in vitro culture. However, while previous
368 researches did not report a significantly different proliferation rate between eBMMSCs and
369 eATMSCs [17] or show a higher doubling time for eATMSCs [8], in the present study cells
370 isolated from equine adipose tissue are characterized by a lower DT compared with cells
371 isolated from equine bone marrow cultured under the same in vitro conditions. Furthermore,
372 different from eBMMSCs, eATMSCs can be grown for longer time in vitro. These
373 characteristics could be very important for using these cells for autologous therapy. Right
374 now, autologous therapy with MSCs is widely used because, as shown by the present study, it
375 does not result in any significant deleterious effects at the time of implantation or later, and
376 shows anti-inflammatory and immunosuppressive effects [23]. However, treatment with
377 autologous MSCs has limitations, such as in acute injuries, because expansion of MSCs by
378 culturing takes different days. Cellular growing data registered by us, similar healing time and
379 no side effects registered in both groups, would make adipose tissue an advantageous source
380 for cellular therapy. Moreover, since no side effects have been observed using these cells for
381 allogenic therapy [unpublished data, 24], it would make adipose tissue also an important
382 MSCs resource for allogenic bank.
383 Minimum criteria for the characterization of human MSCs, postulated by ISCT [5], consider
384 not only the ability of cells to adhere to the plastic when maintained in vitro and their

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

409 30]. Different from that observed in another study [18], in the present research equine MSCs
410 did not display an adipogenic potential lower than other species.
411 As a final step in the differentiation process, we demonstrated that equine MSCs had tri-
412 lineage potential since cells were able to differentiate into chondrocytes. Cells isolated from
413 eAT showed a higher differentiation potency as demonstrated by a greater accumulation of
414 glycosaminoglycans, calcium salt and lipid droplet, comparing with cells isolated from eBM
415 and cultured under the same differentiation condition. These results are not in agreement with
416 those that proved the lack of significant differences between the two lines [31] or reported
417 that osteogenic and chondrogenic differentiation can be better in eBMMSCs [32,33]. Further
418 studies could be conducted to assess the differentiation potential of eAT and eBMMSCs in
419 other lineages of therapeutic interest, such as myocytes, and further investigation are needed
420 using quantitative PCR to confirm a distinct differentiation potential between adipose and
421 bone marrow derived cells.
422 Different from previous studies [18, 31], during the present experiment, the
423 immunophenotypic characterization of the surface of the MSCs used was conducted, by flow
424 cytometry, with the same markers considered in humans, excepted for equine leukocyte
425 antigen-DR surface molecules because of the lack of equine specific monoclonal antibodies
426 available and evidence that certain markers from other species do not cross-react with the
427 equine species [34]. To provide evidence for inter-species cross-reactivity, the similarity of
428 CD markers between human and equine, was identified comparing the amino acid sequence,
429 as suggested by de Mattos Carvalho et al. [35], and we used equine circulating lymphocytes
430 as control [15]. The immunophenotypic investigation was conducted only by flow cytometry,
431 and PCR was not employed. In fact, although this technique shows the mRNA expression of
432 different markers, this expression is not always correlated with the presence of protein,
433 therefore with stemness. However, mRNA expression detection by PCR may possibly

434 complement the results obtained. In agreement with our previous report in horses [15], eBM
435 and eAT, cultured under the same conditions, showed high positivity for CD90 and CD44.
436 CD90, called Thy-1, is an antigen present in established culture of equine MSC. The
437 expression profile of these markers in equine BM- and AT-MSCs at passage 3, was in
438 accordance with the immunophenotype reported for human MSCs by ISCT [5]. The CD44
439 antigen is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and
440 migration. Data observed in the present study confirm those previously reported, by our team,
441 for MSCs isolated from equine foetal adnexa [15]. Relatively low CD105 expression relative
442 to CD90 and CD44 by both MSCs lines has been previously reported in equine by Xie et al
443 [36]. CD105 (endoglin) is a high affinity co-receptor for transforming growth factor (TGF)-β1
444 and TGF-β3 [37]. Although CD105 is generally considered an important marker for MSCs [5]
445 several reports showed that its expression vary depending upon MSC source, culture time in
446 vitro and differentiation state [38,39]. In human and mouse the existence of a heterogeneous
447 cell population CD105 positive and CD105 negative have been recently demonstrated [40,
448 41]. Furthermore, since CD105 is a component of the TGF-β receptor, its presence or absence
449 on the MSCs must have an effect on their response to TGF-β. In particular, MSCs
450 constitutively secrete TGF-β1 in culture and the fetal bovine serum contains high levels of
451 latent TGF-β1 [40, 42], so the expression of this protein could be related to the culture
452 medium composition, and in particular to the presence of serum, as observed recently in
453 human by Mark et al [43]. A lack of reactivity with haematopoietic markers CD14, which
454 cross-reaction was confirmed by lymphocytes investigation, indicates that isolated cells are
455 negative for haematopoietic progenitors. On the other hand isolated cells showed a weak
456 expression of CD34 in both eAT and eBMMSCs, despite in a higher percentage compared
457 with the findings of Ranera et al [9] but without statistically significant differences between
458 the two lineages. Another study on equine MSCs from adipose tissue and bone marrow stated

459 its lack in these lineages [31]. Because the immunoreactivity for CD34 in human AT-MSCs
460 declines with passages [44,45], further analysis is necessary to confirm that the loss of CD34
461 in equine MSCs is similar to that in human cells. Furthermore, the lack of reactivity of equine
462 cells and lymphocytes with the haematopoietic markers CD45 and MSC with marker CD73
463 probably indicates that the human-directed reagents do not cross-react with their
464 corresponding equine epitopes. These findings need further investigation to assess if, in
465 particularly, the lack of CD73 expression is due to the lack of cross-reactivity or is a species-
466 specific feature, due to the same findings in previous studies conducted both on eATMSCs
467 and on foetal MSCs [15, 46]. However, taken together, the results obtained in the present
468 study support an MSC phenotype from both tissue sources used in this investigation.
469 No significant increase in lesion cross-sectional area or pain sensitivity occurred after the
470 implantation of adipose and bone marrow derived MSCs, which is in agreement with the
471 results reported by Fortier and Smith [47], who indicated that the implantation of bone
472 marrow-derived MSCs did not provoke worsening of the lesion or even tendon reaction, with
473 no increase in tendon area in ultrasonographic imaging. The dose of progenitor cells used in
474 this study ($5x10^6$ cells) is lower than that used by other Authors [48]. At present, there are no
475 published studies evaluating the optimal number of MSCs that should be used in the treatment
476 of tendinitis, though one recent report suggests that murine MSCs are potentially cytotoxic
477 when injected in high concentrations directly into tumor tissue (melanoma), liberating several
478 angiogenesis inhibitor agents that induce apoptosis and annul tumor growth, a process that
479 would be of enormous potential in cancer therapy [49]. Whether the administration of high
480 concentrations of MSCs in tendon injuries stimulates the release of angiogenesis inhibitors
481 remains unknown, though this occurs, it could result in the inhibition of tendon healing,
482 which is not desirable. In the present study, the use of eAT and eBMMSCs proved to be safe
483 with the absence of neoplastic tissue formation at the lesion site where the implantation was

484 performed during the experiment. Analysis of the results of the ultrasonographic evaluation of
485 the tendons is in agreement with previously published reports [48]. Furthermore, different
486 from Barreira et al. [50], no ultrasonographic differences in the mean values of the percentage
487 of ruptured collagen fibers in a cross-sectional view have been observed after the
488 administration of MSCs. In our experiment, all ultrasonographic imaging was obtained by the
489 same operator using the same ultrasound equipment, to avoid variation due to different
490 operators and the use of different equipment. This precaution is extremely important because
491 it was demonstrated that significant interoperator variability can occur when measuring the
492 area of the same tendon [51]. Despite the positive results obtained in the present study we are
493 aware that it has some limitations. In the present study owned horses with overstrain SDF
494 injuries have been enrolled, no control group was included neither an animal treated as
495 clinical case has been subjected to histological examination, differently from studies
496 performed in experimental animals [48, 50].
497 Different Authors, for treating induced tendon lesions, performed the implantation of
498 mononucleated cells, derived from adipose and bone marrow tissue, 48 hours after harvest,
499 and they called the mix of cells "stem cells", though MSCs are present in small quantities. In
500 our study, autologous adipose tissue and bone marrow-derived MSCs isolated, expanded in
501 vitro and characterized have been used. Our choice involves greater cost, it is more laborious,
502 and obviously the application requires a delay in the therapy needed for cell expansion in
503 vitro. However, it has an advantage in that the procedure permits isolation and expansion of
504 the number of MSCs, thereby avoiding the administration of a heterogenous cell population
505 that can disturb the process of tendon repair [52].
506 **5. Conclusion**
507 The panel of surface antigens tested by flow cytometry in the present study revealed a similar
508 phenotypic profile between horse and human MSCs, although specific differences in some

509 surface antigens were noticed. A similar cell surface profile was also observed between
510 eBMMSCs and eATMSCs. This findings are important for characterizing these cells before
511 using them for cellular-based therapies in equine medicine. However, many questions still
512 remain, and further investigation will be necessary to clarify the mechanisms and functions of
513 stem cell epitopes, such as the effect of marker expression variation on the pluripotency of
514 MSCs or the study of their expression by cells from different passages. Furthermore, though
515 further investigation are needed using a higher number of animals, our clinical data confirm
516 that eAT and eBMMSCs could be used in clinical trials involving both autologous and
517 allogeneic therapy in horses. Under the experimental conditions of this study, the eATMSCs
518 showed higher in vitro differentiation and cell growth. These findings suggest that eAT may
519 be preferable for cell banking purposes.
520

521 **6. Declaration of interest**

522 The authors declare that there is no conflict of interest that could be perceived as prejudicing
523 the impartiality of the reported research.
524

525 **7. Funding**

526 This research was supported by University of Bologna (RFO: Ricerca Fondamentale
527 Orientata).
528

529 **8. Acknowledgments**

530

531 The authors wish to thank Dr. Silvia Colleoni (AVANTEA Srl, Cremona, Italy) and Prof.
532 Cesare Galli (Department of Veterinary Medical Sciences, University of Bologna, Ozzano
533 Emilia (BO), Italy) for their advice on cell cultures protocols.

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687
688
689

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

697 **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

698

699

700 **Table 4.** Flow cytometry analysis of eATMSCs and eBMMSCs at Passage 3 of in vitro

701 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

702

703

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)

707 **Figure Legends**

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

711

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

715

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

719

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

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