Ultrastructural characteristics and immune profile of equine MSCs from fetal adnexa

1 **Research Article**

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5 ***Equine WJ and AM-MSCs in vitro features***

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19

Abstract

Both in human and equine species, mesenchymal stem cells (MSCs) from amniotic membrane (AM) and Wharton's jelly (WJ), may be particularly useful for immediate use or in later stages of life, after cryopreservation in cell bank. The aim of this study was to compare equine AM- and WJ-MSCs *in vitro* features, that may be relevant for their clinical employment. MSCs were more easily isolated from WJ, even if MSCs derived from AM exhibited most rapid proliferation ($P<0.05$). Osteogenic and chondrogenic differentiation was most prominent in MSCs derived from WJ, as also suggested by the lower adhesion of AM cells, demonstrated by the greater volume of spheroids after hanging drop culture ($P<0.05$). Data obtained by PCR confirmed the immunosuppressive function of AM and WJ-MSCs and the presence of active genes specific for anti-inflammatory and angiogenic factors (IL-6, IL 8, IL-β1). For the first time, by means of transmission electron microscopy (TEM), we ascertained that equine WJ-MSCs constitutively contain a very impressive number of large vesicular structures, scattered throughout the cytoplasm and there was an abundant extracellular fibrillar matrix located in the intercellular spaces among WJ-MSCs. Results reveal that MSCs from different fetal tissues have different characteristics that may drive their therapeutic use. Data recorded in this study could be noteworthy for horses as well as for other mammalian species, including humans.

Keywords:

Mesenchymal stem cells, amniotic membrane, Wharton's jelly, equine, electron microscopy

42 **Introduction**

43 Mesenchymal stem cells (MSCs) are a population of multipotent stem cells and since their
44 properties, MSCs offer a great chance for cell-based therapies and tissue engineering
45 applications. Bone marrow (BM) is the common source of autologous MSCs for clinical
46 applications in equine medicine. Alternatively, adipose tissue-derived MSCs can be used,
47 since they have a higher proliferation potential (Iacono *et al.*, 2015a). Anyway, for both
48 sources, an invasive procedure is required and there is a large variability in the cell yield
49 related to the donor (Colleoni *et al.,* 2009). Furthermore, although bone marrow is the most
50 widely investigated source of MSCs, these have limited potential in terms of in vitro
51 proliferation capability (Guest *et al.,* 2010; Lange-Consiglio *et al.,* 2013), and do not appear
52 to noticeably improve long-term functionality compared to those from extra-fetal tissues
53 (Paris and Stout, 2010). Placental tissues and foetal fluids represent a source of cells for
54 regenerative medicine, and are readily available and easily procured without invasive
55 procedures. MSCs from foetal fluids and adnexa are defined as an intermediate between
56 embryonic and adult SCs, due to the preservation of some characteristics typical of the
57 primitive native layers (De Coppi *et al*., 2007). Among foetal adnexal tissues, the major
58 sources of MSCs are amniotic membrane and Wharton's Jelly (Iacono *et al.,* 2015b).
59 Althought, the increasing interest in using MSCs for regenerative medicine in horses, and the
60 possibility to employ MSCs from perinatal tissue both for immediate use in newborns, both in
61 later stages of life, after cryopreservation in cell bank, there is lacking of information on
62 comparison between equine MSCs derived from AM and WJ were compared.

63 Usually, clinical treatments with MSCs are based on their transplantation but only a small
64 percentage of the injected MSCs engraft successfully (Chimenti *et al*., 2010). Consistent with
65 these findings, some studies recently showed that the regenerative ability of MSCs could be
66 attributed to the production of molecules and mediators capable of activating the intrinsic

67 repair processes in the damaged tissues. Different Authors, working on cardiac, renal, spinal
68 cord and tendon regeneration, indicate that the beneficial effects of MSCs can be attributed to
69 the activation of paracrine mechanisms enabling stimulation of endogenous stem cells. These
70 cells are responsible for the bioactive soluble factors (lipids, growth factors, and cytokines)
71 known to inhibit apoptosis and fibrosis, enhance angiogenesis, stimulate mitosis and/or
72 differentiation of tissue-resident progenitor cells, and modulate the immune response (Yagi et
73 *al.*, 2010; Liang *et al.*, 2014). Recently, the ability of equine adult MSCs to secrete numerous
74 soluble mediators, implicated in the inhibition of T-cell proliferation, when stimulate with
75 INF-gamma and TNG-alpha, was demonstrated (Carrade *et al.*, 2012; Kol *et al*., 2013).
76 However, to our knowledge, no studies are present on the immunophenotype profile, before in
77 vitro stimulation, of equine WJ-MSCs and AM-MSCs, to better know their role in the
78 immune response, angiogenesis, apoptosis, oxidation level and cell migration. Furthermore, in
79 addition to soluble factors, recent findings indicate that extracellular vesicles are released
80 from MSCs inside the CM and that these can be involved as important mediators in cell- to-
81 cell communication (Pascucci *et al.*, 2014; Pascucci *et al.*, 2015). Microvesicles (MVs) have
82 been categorized into exosomes (EXs), released from the endosomal compartment, and
83 shedding vesicles (SVs), which bud directly from the cell membrane (Biancone *et al.*, 2012).
84 MVs seem to be involved in a dynamic mutual paracrine communication between the
85 embryonic and the maternal environment at the early stage of pre-implantation embryo
86 development (Saadeldin *et al*., 2015). Recently, Lange-Consiglio *et al.* (2016) and Perrini *et*
87 *al.* (2016) identified the presence and type of MVs secreted by equine AM-MSCs; the
88 Authors also evaluated, in a preliminary study in vitro, the possible therapeutic implication of
89 MVs in endometrial and tendon pathologies. Despite these studies on equine AM-MSCs, the
90 recognized importance of WJ as an alternative source of MSCs both in equine and human
91 medicine (Iacono *et al.*, 2012; Subramanian *et al.*, 2015) and despite a lot of data have been

92 reported on these features of equine adult MSCs (Pascucci *et al.,* 2010; Maia *et al.,* 2013;
93 Pascucci *et al*., 2014; Pascucci *et al.,* 2015), no studies are present on ultrastructural
94 characteristics and MVs of equine WJ-MSCs. In this context, the aims of the present study
95 were to analyze expression of stemness markers, immunophenotype, and ultrastructural
96 features. In addition, we considered migration and adhesion ability of equine WJ-MSCs and
97 AM-MSCs since both the migration ability, the expression of adhesion molecules and the
98 homing to injured environments are important features of MSCs (Burk *et al.*, 2013; Kavanagh
99 *et al.*, 2014).
100
101 **Materials and Methods**
102
103 *Materials*
104 Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (St. Louis, MO,
105 USA), and laboratory plastics from Sarstedt Inc. (Newton, NC, USA).
106
107 *Animals*
108 Samples were recovered from 13 Standardbred mares, housed at the Department of Veterinary
109 Medical Sciences, University of Bologna, for attended delivery. Experimental procedures
110 were approved by the Ethics Committee, University of Bologna (8134-X/10). The written
111 consent was given by the owners to allow tissues recovery for research purposes.
112
113 *Umbilical cord collection and WJ-MSCs isolation*
114 Immediately after breaking the umbilical cord (UC), the part closest to the colt, characterized
115 by an abundant amount of WJ, was severed. For avoiding mildew and bacterial
116 contamination, samples were washed under flowing water for removing straw or feces debris.

117 Until processing, samples were stored in D-PBS (Dulbecco's Phosphatase Buffered Solution)
118 containing penicillin (100 IU/ml) and streptomycin (100 mg/ml), at 4°C for at the latest 12 h.
119 In the lab, before WJ enzymatic digestion, under a laminal flow hood, UCs were disinfected
120 by immersing for few seconds in 70% ethanol and rinsed by repeated immersion in D-PBS.
121 WJ was then isolated, weighed, minced finely (0.5 cm^2) by sterile scissors and cells were
122 isolated as previously described (Iacono *et al.*, 2012). Briefly, WJ fragments were incubated
123 in a 37 °C water bath for 1-2 h into a 50ml polypropylene tube, containing 1ml/1g sample of
124 digestion solution (0.1% (w/v) collagenase type I (Gibco, Invitrogen Corporation, Carlsbad,
125 California, USA), in D-PBS). The mixture was then filtered to separate the dispersed amnion
126 cells from the tissue pieces and collagenase was inactivated by diluting 1:1 with D-PBS plus
127 10% (v/v) FBS (Gibco). Nucleated cells were pelleted at 470 g for 10 min. The supernatant
128 was discarded, pellet was re-suspended in 5 ml of culture medium (Dulbecco's Modified
129 Essential Medium (D-MEM)-F12 Glutamax® (Gibco) supplemented with 10% v/v FBS, 100
130 iu/ml penicillin and 100 µg/ml streptomycin) and spun at 470 g for 10 min to wash cells. This
131 operation was repeated three times. After the last wash, cell pellet was re-suspended in 1 ml
132 of culture medium and cell concentration was determined by haemocytometer.

133

134 *Amnion collection and cells isolation*

135 Allanto-amniotic membranes were obtained at pregnancy term, after vaginal delivery.
136 Portions of allanto-amnion were washed under flowing water for removing straw or feces
137 debris, stored at 4°C in D-PBS, added with antibiotics (100 iu/ml penicillin and 100 μg/ml
138 streptomycin), and were processed within 12h. In the lab, before enzymatic digestion, under a
139 laminal flow hood, samples were disinfected by immersing for few seconds in 70% ethanol
140 and rinsed by repeated immersion in D-PBS. Then, AM was stripped from the overlying
141 allantois, weighted and cut into small pieces (0.5 cm^2) by sterile scissors. Cells were then

142 isolated as described above for WJ, by an enzymatic digestion.

143

144 *Cell culture and proliferation assays*

145

146 After isolation, primary cells derived from all recovered samples were plated in a 25 cm^2 flask
147 in 5 ml of D-MEM-F12 Glutamax®, plus 10% v/v FBS and antibiotics. Cells were incubated
148 in a 5% CO_2 humidified atmosphere at 38.5°C. At ~80-90% of confluence, they were
149 dissociated by 0.25% trypsin, counted and plated at the concentration of $5x10^3$ cells/cm^2 as
150 "Passage 1" (P1), and so on for the following passages. Calculation of cell-doubling time
151 (DT) and cell-doubling numbers (CD) was carried out according to the formulae of (Rainaldi
152 *et al.*, 1991):

153 $$CD= \ln(Nf/Ni)/\ln(2) \quad (1)$$

154 $$DT= CT/CD \quad (2)$$

155 where Nf is the final number of cells and Ni the initial number of cells.

156

157 *Adhesion and Migration Assays*

158 To define differences between WJ and AM-MSCs, spheroid formation and migration test
159 were performed. Three replicates for each experiment were performed; all replicates were
160 carried out at passage 3 of *in vitro* culture.

161 For adhesion assay, cells were cultured in 'hanging drops' (5.000 cells/drop of 25μl) for 24 h.
162 Images were acquired by a Nikon Eclipse TE 2000-U microscope. Spheroid areas were
163 determined using ImageJ software (imagej.nih.gov/ij/). Starting from the binary masks
164 obtained by Image J, the volume of each spheroid was computed using ReViSP
165 (sourceforge.net/projects/revisp) (Bellotti *et al.*, 2016), a software specifically designed to
166 accurately estimate the volume of spheroids and to render an image of their 3D surface.

167 To assess cell migration potential, a scratch assay (also known as Wound-Healing assay) was
168 carried out, as previously described (Liang *et al.*, 2007). Briefly, at 80–90 % confluence the
169 cell monolayer was scraped using a p1000 pipet tip. After washing twice with D-PBS, the
170 dish was incubated for 24 h at 38.5 °C and 5 % CO2 in a humidified atmosphere. Images were
171 acquired both immediately after the tip-scratch (time 0; T0) and after the incubation period
172 (last time point or time 1; T1), and the distances of each scratch closure were calculated by
173 ImageJ software. The migration percentages were calculated using the following formula
174 (Rossi *et al.*, 2014):

175 [(distance at T0-distance at T1)*100]/distance at T0

176

177 *In vitro differentiation*

178 *In vitro* differentiation potential of cells towards osteogenic, adipogenic and chondrogenic
179 lineages was studied. Cells ($5x10^3$ cells /cm^2) were cultured under specific induction media
180 (Table 1). As negative control, an equal number of cells was cultured in expansion medium.
181 *In vitro* differentiation potential was assessed at passage 3 of culture in two replicates for 3
182 samples from each lineage. To cytologically evaluate differentiation, cells were fixed with
183 10% formalin at room temperature (RT) and stained with Oil Red O, Alcian Blue and Von
184 Kossa for adipogenic, chondrogenic and osteogenic induction, respectively. Quantitative
185 analysis of *in vitro* differentiation was performed by Image J.

186

187 *Immunocytochemistry (ICC)*

188 Cells, derived from 3 AM and WJ samples, at P3, were cultured on ICC slides, until
189 confluence. They were then fixed with 4 % paraphormaldehyde (20 min at RT) and then
190 washed in phosphate buffer (PB). Cells were blocked in goat serum (10 %) for 1 h and
191 incubated overnight with primary antibodies (Table 2); the day after, they were washed in
192 PB2 (PB + 0.2 % BSA + 0.05 % saponin) and incubated with anti-mouse- or anti-rabbit-
193 FITC conjugated secondary antibodies for 1 h. Nuclei were then labelled with Hoechst 33342.
194 The excess of secondary antibody and Hoechst were removed by 3 washes with PB2. Images
195 were obtained with a Nikon Eclipse E400 microscope, using the software Nikon NIS-
196 Elements.

197

198 *Molecular Characterization*

199 To evaluate pluripotency potential of the two types of equine cells, PCR for the pluripotency
200 genes OCT4, NANOG and SOX2 was performed. Gene expression was tested on equine
201 blastocysts, as positive control. To test cell stemness and immunoproperty, the following set

202 of genes was evaluated: CD45, CD 34, CD90, CD73, MHC-I, MHC-II, IL-β1, IL-4, IL-6, IL-
203 8, INF-γ, TNF-α. Primers were tested on activated equine lymphocyte. The specific set of
204 primers used is listed in Table 3. All tests were carried out on $100x10^3$ cells, derived from
205 AM and WJ of three different mares. Experiments were performed at passage 3 of culture.
206 Cells were snap-frozen and RNA was extracted using Nucleo Spin® RNA kit (Macherey-
207 Nagel, Düren, Germany) following the manufacturer's instructions. cDNAs were synthesized
208 by RevertAid RT Kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) and used
209 directly in PCR reactions, following the instructions of Maxima Hot Start PCR Master Mix
210 (2X) (ThermoFisher Scientific, Waltham, Massachusetts, USA). PCR products were
211 visualized with ethidium bromide on a 2% agarose gel.
212
213 *Transmission Electron Microscopy (TEM)*
214 Ultrastructural examination was performed on AM (n=3) and WJ-MSCs (n=3) at P3. The
215 analysis was performed on three replicates. After detaching cells, the pellet was fixed with
216 2.5% glutaraldehyde in 0.1 M PB, pH 7.3, for 1 h, at RT. Cells were then washed twice in PB
217 and post-fixed with buffered 2% osmium tetroxide for 1 h, at RT. They were finally
218 dehydrated in a graded ethanol-propylen oxide series, pre-infiltrated and embedded in Epon
219 812. Ultrathin sections (90 nm) were mounted on 200-mesh copper grids, stained with uranyl
220 acetate and lead citrate, and examined by a Philips EM 208 microscope, equipped with a
221 digital camera (Center for Electron Microscopy, CUME, University of Perugia).
222
223 *Statistical Analysis*
224 Harvested WJ and AM (grams), CDs, DTs and percentages of migration are expressed as
225 mean ± standard deviation (SD). Statistical analyses were performed using IBM SPSS
226 Statistics 21 (IBM Corporation, Armonk, New York, USA). Data were analysed, for normal

227 distribution, using a Shapiro-Wilk test, then using one-way ANOVA or a Student's t-test
228 (CDs and DTs). The 3D spheroid volumes and mean grey intensity of differentiated cells
229 were compared using Mann-Whithney's U-test, due to their non-normal distribution.
230 Significance was assessed for $P<0.05$.
231
232 **Results**
233
234 *Cellular Growth*
235 As soon as after foals' birth and immediately after foal detachment, UC (length ~15 cm) and
236 AM samples were recovered. The mean weight of recovered jelly and AM were 5.22±3.34 g
237 and 15.60±5.23 g, respectively. Adherent mononuclear cells, characterized by elongated
238 fibroblast-like morphology were isolated in 13/13 (100%) WJ samples and in 9/13 (69.2%)
239 AM samples. Undifferentiated cells of both lines were passaged up to seven times; no
240 changes in cell morphology was observed throughout the culture period. DTs assay showed
241 that AM and WJ-MSCs were able to divide for an extensive period *in vitro*. During P0 to P7,
242 AM-MSCs showed a mean DT of 1.49±0.34 days/CD, significantly lower than that recorded
243 for WJ-MSCs (1.71±0.65 days/CD; $P<0.05$). No statistically significant differences were
244 found in DTs among earlier culture passages in both cell lines ($P>0.05$). However, AM-MSCs
245 start to grow more slowly by P6, a sign of cellular aging; on the contrary, WJ-MSCs, despite
246 an higher DT, get older later, starting from P7. This is confirmed by significantly higher DTs
247 compared to the earliest steps ($P<0.05$). By P7, total WJ and AM-MSCs cell doublings were
248 similar (36.57±0.76 vs 37.05±0.59; $P>0.05$; Fig.1).
249
250 *Adhesion and Migration Assays*

251 Both AM and WJ cells formed spheroids when cultured in hanging drops. Average areas and
252 volume of the spheroids formed by WJ-MSCs were significantly smaller than from AM-
253 MSCs ($P<0.05$; Fig.2). Average percentage of migration, observed by scratch test, was
254 statistically similar between cell lines (AM-MSCs vs WJ-MSCs: 34.14±4.51% vs
255 38.20±2.88%; $P>0.05$; Fig. 2).
256
257 *In vitro Differentiation*
258 Both cell lines were able to differentiate toward osteogenic, chondrogenic and adipogenic
259 direction (Fig.3). However, WJ-MSCs showed a greater chondrogenic and osteogenic
260 potential ($P<0.05$), characterized by a greater accumulation of extra-cellular mucosubstances
261 and calcium deposits, as showed by Alcian Blu (Fig. 3A) and Von Kossa (Fig.3B) stains
262 respectively.
263
264 *Immunostaining and PCR analysis*
265 Immunostaining results are showed in Figure 4. Amniotic membrane and WJ MSCs clearly
266 expressed mesenchymal marker, N-Cadherin, and the mesodermal marker alpha-SMA. On the
267 contrary, they did not express pan-cytokeratin and E-Cadherin.
268 PCR results are reported in Table 4; positive expression are also showed in Figure 5. Both cell
269 populations expressed MSC-associated markers (CD90, CD73), while were negative for an
270 hematopoietic marker (CD45), at P3 of *in vitro* culture. On the contrary, the haematopoietic
271 marker CD34 was registered for either population. Both WJ-MSCs and AMSCs lacked MHC-
272 I and MHC-II expression. Regarding embryonic markers, WJ-MSCs expressed OCT-4, while
273 AM-MSCs were weakly positive for this marker; both cell populations lacked Nanog and
274 Sox2. About their immune-phenotype, both WJ-MSCs and AMSCs lacked MHC-I, MHC-II,

275 INF- γ, TNF-α and IL-4 expression. Cell were instead positive for IL-6 and IL- β1. WJ-MSCs
276 expressed IL-8 marker, while a weak expression was showed by AM-MSCs.
277
278 *TEM*
279 At low magnification, cells of both samples were quite small and uniform in size (diameter
280 range: 10-15 μm; Fig. 6A; Fig. 7A). AM-MSCs appeared generally well dissociated, while
281 WJ MSCs were frequently tightly adherent each other to form wide aggregates (Fig. 6A and
282 B). Golgi complex was particularly well developed; it occupied a juxta-nuclear position and
283 exhibited flattened cisternae, transport vesicles, and heterogeneous sized secreting granules.
284 Some of them were very large and enclosed fine granular material (Fig. 6C and D). RER
285 showed linear flat profiles and dilated cisternae (Fig. 6E and F). In both samples, the most
286 interesting ultrastructural feature was represented by the very impressive number of large
287 vesicular structures, up to 2 μm in diameter, scattered throughout the cytoplasm (Fig. 6G and
288 H). They showed a variety of appearances and ranged from multivesicular bodies (MVB)
289 (Fig. 7A and B) comprising intralumenal nanovesicles of different sizes (30-500 nm), to
290 endolysosomes and autophagic vacuoles. They were particularly abundant in WJ-MSCs. The
291 occurrence of membrane vesicles shedding from cell surface was observed in both samples.
292 They ranged in size from 100 nm to 500 nm and included electron-lucent, as well as
293 moderately electron-dense vesicles isolated or aggregated nearby the cells (Fig. 7C and D).
294 Complex extracellular vesicles measuring 500nm-1μm and containing packed nanovesicles,
295 frequently budded from the cell surface or were detected in the intercellular space (Fig. 7E
296 and F). Tunneling nanotubes were occasionally observed in both samples suggesting that this
297 may be an additional mechanism of crosstalk between MSCs (Fig. 7G and H). The most
298 noteworthy difference between AM-MSCs and WJ-MSCs was the presence of an abundant
299 extracellular fibrillar matrix (EFM) located in the intercellular spaces among WJ-MSCs (Fig.

300 8A-C). It was composed of a finely granular and moderately electron-dense ground substance
301 populated by a loosely arranged network of reticular fibrils. These were uniformly thin and
302 tend to run parallel to the cell surface. Abundant vesicles were entrapped among the fibrils
303 (Fig. 8C). The intercellular spaces were devoid of collagen fibrils.
304
305 **Discussion**
306 AM-MSCs and WJ-MSCs are the focus of great interest in human and veterinary regenerative
307 medicine for their in vitro multilineage differentiation potential, their great in vitro expansion
308 (Iacono *et al.,* 2012; Lange-Consiglio *et al.*, 2013). In the present study, for the first time in
309 equine species, proliferation, migration, spheroids formation, trilineage differentiation
310 capacity, expression of stemness markers, immunophenotype and ultrastructural features of
311 MSCs derived from WJ and AM were compared. From both tissues, cells with mesenchymal
312 morphology were isolated. However, as recently reported in human (Subramanian *et al.*,
313 2015), in the present study, MSCs were isolated from all samples by collagenase digestion
314 technique only for WJ. No other reports exist on the successful isolation rate from equine WJ
315 and AM. Despite the lower isolation rate, AM-MSCs showed a higher proliferation rate
316 compared to WJ-MSCs. As in human (Pasquinelli *et al.*, 2007), in both cell types, TEM
317 examination revealed an highly metabolic and synthetic nature, demonstrated by euchromatic
318 nucleus, prominent nucleoli, abundant nuclear pores as well as by well-developed RER and
319 Golgi complex. Furthermore, the higher DTs were unrelated with total cell doubling number,
320 because AM-MSCs began to grow old earlier, as registered by a higher DT at P6 of in vitro
321 culture, confirming the proliferative nature of WJ-MSCs. Beyond the growth curve, migration
322 ability is an important feature of MSCs because of its fundamental significance for systemic
323 application (Li *et al.*, 2009; Burk *et al.*, 2013). No differences were found between WJ-MSCs
324 and AM-MSCs in migration ability. Since the adhesion capability is related and enhanced to

325 differentiation potential (Pasquinelli *et al.*, 2007; Wang *et al.*, 2009; Kavanagh *et al.*, 2014),
326 in the present study spheroid formation *in vitro* was assessed using the hanging drop method.
327 Cell derived from WJ showed a higher adhesion ability, forming smaller spheroids, as
328 determined by ReVisp. In the present study, the analysis of differentiated cells by Image J
329 showed a higher WJ-MSCs chondrogenic and osteogenic potential. Our results confirmed
330 data recently registered with human WJ-MSCs and AM-MSCs cell (Subramanian *et al.* 2015),
331 in fact also equine WJ-MSCs, exposed to osteocyte and chondrocyte differentiation media,
332 showed the highest number of Von Kossa stained cells, greatest staining intensity of nodules
333 and higher number of cells positive for Alcian Blue compared to cells from AM
334 (Subramanian *et al.*, 2015). Besides to differentiation ability, the equine fetal adnexa derived
335 MSCs demonstrate the characteristics defined by the International Society for Cellular
336 Therapy criteria (Dominici *et al.*, 2006), except for the CD34. CD34 is predominantly
337 regarded as a marker of hematopoietic stem cells (HSC) and hematopoietic progenitor cells.
338 Accumulating evidence demonstrates CD34 expression on several other cell types, including
339 embryonic stem cell derived MSC (Kopher *et al.*, 2010) and multipotent mesenchymal
340 stromal cells (MSC) (Nielsen and McNagny, 2008). In many cases, CD34 indicate a distinct
341 subset of cells with enhanced progenitor activity (Sidney *et al.*, 2014). The expression of
342 CD34 by equine cells might constitute evidence of their potenciality. Moreover, as
343 intermediate between adult and embryonic cells, equine WJ and AM-MSCs express OCT-4, a
344 marker for pluripotent stem cells. However, as previously reported in human (Subramanian *et*
345 *al.*, 2015), also in equine, the expression level of OCT-4 seems to be lower for cells from AM
346 compared to WJ. This finding, coupled with greater differentiation ability, could be related to
347 the middle position of WJ between blastocyst and adult. The stem cells isolated from the WJ
348 probably start to lose their embryonic pluripotency tumorigenic characteristics and start to
349 acquire multipotent non-tumorigenic MSC characteristics with progressive development. This

350 feature would help cells from the WJ to differentiate into specific lineages more easily both in
351 vitro and during cell-based therapy and allow higher reprogramming efficiency to the
352 embryonic state because of an immature phenotype (Pera *et al*., 2009). In human cells derived
353 from WJ, the telomerase levels remained high throughout serial culture compared to AM-
354 MSCs suggesting that they retain their primitive characteristics in culture for long periods of
355 time (Subramanian *et al.*, 2015). In equine species further studies are needed to verify this
356 condition.
357 Due to the importance of MSCs for their immune response and their ability to suppress T-
358 cells (Carrade *et al.*, 2012), in the present study, anti-inflammatory and pro-inflammatory
359 factors produced both by WJ-MSCs and AM-MSCs were investigated for the first time in the
360 horse. One of the most important cytokines of the acute phase reaction is TNF-α, while IL-4
361 is a cytokine involved in allergic inflammation. Different from that observed in human cells,
362 equine WJ and AM-MSCs do not express these markers, neither INF-γ when they are not
363 stimulate in vitro by the presence of INF. Confirming their reduced immunogenicity, both cell
364 lines were negative for MHC-I and MHC-II. On the contrary, both cell lines expressed, on
365 their cDNA, IL-1β, IL-6 and IL-8; this cytokines are important mediator of the inflammatory
366 response, involved in a variety of cellular activities, including cell proliferation,
367 differentiation, apoptosis, chemotaxis, angiogenesis and hematopoiesis (Lamalice *et al.*,
368 2007). Data registered in this study confirmed those already reported in human WJ-MSCs
369 (Dominici *et al.*, 2006; Choi *et al.*, 2013) and AM-MSCs (Yazdanpanah *et al.*, 2015). These
370 factors are involved in the complex interaction between MSCs and the tissue
371 microenvironment as well as in the production of membrane vesicles, containing molecules
372 such as short peptides, proteins, lipids, and various forms of RNAs (György *et al.*, 2011). As
373 previously observed in adult equine cells (Pascucci *et al.*, 2014), the great number of MVB
374 that, contained intralumenal vesicles maturing from their internal membrane, may be

375 interpreted as the ability of both cell types to produce a huge variety of "secreting" molecules
376 enclosed inside vesicles of different types that are released in the extracellular milieu. Maybe
377 hypothesized, in addition, that the several other vesicular structures observed by TEM
378 represent a mechanism to efficiently recycle cell constituents by autophagy. The intense
379 proliferating and metabolic activity, in fact, makes it necessary to constantly renew sub-
380 cellular components, especially membrane fractions. The main difference between AM-MSCs
381 and WJ-MSCs attained the presence of an abundant extracellular fibrillar matrix in the
382 intercellular spaces among WJ-MSCs; it probably determines a tight intercellular adhesion
383 even after trypsin treatment and is responsible for the observation of cell aggregates at TEM
384 analysis. It is well known that these cells, in vivo, are immersed in a mucoid connective
385 matrix. It seems evident that WJ-MSC isolation and cultivation in vitro does not affect their
386 ability to produce extracellular matrix.
387
388 **Conclusion**
389 It has emerged from the present study that cells isolated from different fetal origin matrices
390 exhibit different morphological, molecular and differentiation potential. Equine WJ could be
391 considered as a viable source for MSCs with reliable migration and differentiation capacities,
392 and it is therefore a convenient cell source for autologous or allogeneic regenerative therapies.
393 Although the molecular content and functional activities of EVs produced by WJ and AM-
394 MSCs remain to be characterized, the results of the present study indicated that MSCs from
395 equine fetal adnexa are able to constitutively produce EVs that may be partly responsible for
396 their paracrine activity. Further investigation are needed to find the best protocols for isolation
397 and *in vitro* differentiation for AM-MSCs. Moreover, additional *in vivo* tests are needed to
398 confirm our *in vitro* findings.
399

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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