1 **Research Article**

2

3 **Ultrastructural characteristics and immune profile of equine MSCs from fetal adnexa**

4

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

6

7 Eleonora Iacono[a*], Luisa Pascucci[b], Barbara Rossi[a], Cinzia Bazzucchi[b], Aliai Lanci[a], Monica

8 Ceccoli[a], Barbara Merlo[a]

9

10 [a] *Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50,*

11 *40064 Ozzano Emilia (BO), Italy.*

12 [b] *Department of Veterinary Medicine, University of Perugia, via San Costanzo 4, 06126*

13 *Perugia, Italy.*

14

15 *Corresponding author: Prof. E. Iacono, Department of Veterinary Medical Sciences,

16 University of Bologna, via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy.

17 Tel.: +39 051 2097567

18 E-mail address: eleonora.iacono2@unibo.it

19

20 **Abstract**

21

22 Both in human and equine species, mesenchymal stem cells (MSCs) from amniotic membrane

23 (AM) and Wharton's jelly (WJ), may be particularly useful for immediate use or in later

24 stages of life, after cryopreservation in cell bank. The aim of this study was to compare equine

25 AM- and WJ-MSCs *in vitro* features, that may be relevant for their clinical employment.

26 MSCs were more easily isolated from WJ, even if MSCs derived from AM exhibited most

27 rapid proliferation ($P<0.05$). Osteogenic and chondrogenic differentiation was most

28 prominent in MSCs derived from WJ, as also suggested by the lower adhesion of AM cells,

29 demonstrated by the greater volume of spheroids after hanging drop culture ($P<0.05$). Data

30 obtained by PCR confirmed the immunosuppressive function of AM and WJ-MSCs and the

31 presence of active genes specific for anti-inflammatory and angiogenic factors (IL-6, IL 8, IL-

32 $\beta 1$). For the first time, by means of transmission electron microscopy (TEM), we ascertained

33 that equine WJ-MSCs constitutively contain a very impressive number of large vesicular

34 structures, scattered throughout the cytoplasm and there was an abundant extracellular

35 fibrillar matrix located in the intercellular spaces among WJ-MSCs. Results reveal that MSCs

36 from different fetal tissues have different characteristics that may drive their therapeutic use.

37 Data recorded in this study could be noteworthy for horses as well as for other mammalian

38 species, including humans.

39 *Keywords:*

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

41

Introduction

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

Usually, clinical treatments with MSCs are based on their transplantation but only a small percentage of the injected MSCs engraft successfully (Chimenti *et al*., 2010). Consistent with these findings, some studies recently showed that the regenerative ability of MSCs could be 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)   (1)

154   DT= CT/CD   (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 % CO_2 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

References

Bellotti C, Duchi S, Bevilacqua A, Lucarelli E & Piccinini F 2016 Long term morphological characterization of mesenchymal stromal cells 3D spheroids built with a rapid method based on entry-level equipment. *Cytotechnology* **68** 2479–2490.

Biancone L, Bruno S, Deregibus MC, Tetta C & Camussi G 2012 Therapeutic potential of mesenchymal stem cell-derived microvesicles. *Nephrology Dialisis Transplantation* 27 3037–3042.

Burk J, Ribitsch I, Gittel C, Juelke H, Kasper C, Staszyk C & Brehm W 2013 Growth and differentiation characteristics of equine mesenchymal stromal cells derived from different sources. *The Veterinary Journal* **195** 98–106.

Carrade DD, **Lame MW, Kent MS, Clark KC, Walker NJ & Borjesson DL** 2012 Comparative analysis of the immunomodulatory properties of Equine Adult-Derived Mesenchymal Stem Cells. *Cell Medicine* **4** 1-11.

Castagnetti C, Mariella J, Pirrone A, Cinotti S, Mari G & Peli A 2012 Expression of interleukin-1 β, interleukin-8, and interferon-γ in blood samples obtained from healthy and sick neonatal foals. *American Journal of Veterinary Research* **73** 1418–1427.

Chimenti I, Smith RR, Li TS, Gerstenblith G, Messina E, Giacomello A & Marbán E 2016 Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. *Circulation Research* **106** 971–980.

Choi M, Lee H, Naidansaren P, Kim H, Cha J, Ahn H, Yang P, Shin J & Joe Y 2013 Proangiogenic features of Wharton's jelly-derived mesenchymal stromal/stem cells and their ability to form functional vessels. *The International Journal of Biochemistry and Cell Biology* **45** 560–570.

Colleoni S, Bottani E, Tessaro I, Mari G, Merlo B, Romagnoli N, Spadari A, Galli C & Lazzari G 2009 Isolation, growth and differentiation of equine mesenchymal stem cells:

440 effect of door, source, amount of tissue and supplementation with basic fibroblast growth

441 factor. *Veterinary Research Communication* **33** 811-821.

442 **Corradetti B, Lange-Consiglio A, Barucca M, Cremonesi F & Bizzarro D** 2011 Size-

443 sieved subpopulations of mesenchymal stem cells from intervascular and perivascular equine

444 umbilical cord matrix. *Cell Proliferation* **44** 330–342.

445 **De Coppi P, Bartsch G, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre**

446 **AC, Snyder EY, Yoo JJ, Furth ME, Soker S & Atala A** 2007 Isolation of amniotic stem

447 cell lines with potential for therapy. *Nature Biotechnology* **25** 100–106.

448 **Desmarais J, Demers S, Suzuki JJ, Laflamme S, Vincent P, Laverty S & Smith L** 2011

449 Trophoblast stem cell marker gene expression in inner cell mass-derived cells from

450 parthenogenetic equine embryos. *Reproduction (Cambridge, England)* **141** 321–332.

451 **Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R,**

452 **Keating A, Prockop D & Horwitz E** 2006 Minimal criteria for defining multipotent

453 mesenchymal stromal cells. The International Society for Cellular Therapy position statement.

454 *Cytotherapy* **8** 315–317.

455 **Guest DJ, Smith MR & Allen WR** 2010 Equine embryonic stem-like cells and

456 mesenchymal stromal cells have different survival rates and migration patterns following their

457 injection into damaged superficial digital flexor tendon. *The Equine Veterinary Journal* **42**

458 636–642.

459 **György B, Szabò T, Pàsztòi M, Pàl Z, Mijàk P, Aradi B, Làszlò V, Pàllinger E, Pap E,**

460 **Kittel A** *et al.* 2011 Membrane vesicles, current state-of-the-art: emerging role of

461 extracellular vesicles. *Cellular and Molecular Life Sciences* **68** 2667–2688.

462 **Iacono E, Brunori L, Pirrone A, Pagliaro PP, Ricci F, Tazzari PL & Merlo B** 2012

463 Isolation, characterization and differentiation of mesenchymal stem cells from amniotic fluid,

umbilical cord blood and Wharton's jelly in the horse. *Reproduction (Cambridge, England)* **143** 455–468.

Iacono E, Merlo B, Romagnoli N, Rossi B, Ricci F & Spadari A 2015a Equine bone marrow and adipose tissue mesenchymal stem cells: Cytofluorimetric characterization, in vitro differetiation and clinical application. *Journal of Equine Veterinary Sciences* **35** 130-140.

Iacono E, Rossi B & Merlo B 2015b Stem cells from foetal adnexa and fluid in domestic animals: an update on their features and clinical application. *Reproduction in Domestic Animals* **50** 353-364.

Jischa S, Walter I, Nowotny N, Palm F, Budik S, Kolodziejek J & Aurich C 2008 Uterine involution and endometrial function in postpartum pony mares. *American Journal of Veterinary Research* **69** 1525–1534.

Kavanagh D, Robinson J & Kalia N 2014 Mesenchymal stem cell priming: fine-tuning adhesion and function. *Stem Cell Reviews* **10** 587–599.

Kol A, Walker NJ, Galuppo LD, Clark KC, Buerchler S, Bernanke A & Borjesson DL 2013 Autologous point-of-care cellular therapies variably induce **equine**mesenchymal stem cell migration, proliferation and cytokine expression. *The Equine Veterinary Journal* **45** 193-198.

Kopher RA, Penchev VR, Islam MS, Hill KL, Khosla S & Kaufman DS 2010 Human embryonic stem cell-derived CD34+ cells function as MSC progenitor cells. *Bone* **47** 718–728.

Lamalice L, Le Boeuf F & Huot J 2007 Endothelial cell migration during angiogenesis. *Circulation Research* **100** 782–794.

Lange-Consiglio A, Corradetti B, Bizzaro D, Magatti M, Ressel L, Tassan S & Parolini O, Cremonesi F 2012 Characterization and potential applications of progenitor cells isolated

from horse amniotic membrane. *Journal Tissue Engineering Regenerative Medicine* **6** 622-635.

Lange-Consiglio A, Corradetti B, Meucci A, Bizzaro D & Cremonesi F 2013 Characteristics of equine mesenchymal stem cells derived from amnion and bone marrow: in vitro proliferative and multilineage potential assessment. *The Equine Veterinary Journal* **45** 737–744.

Lange-Consiglio A, Perrini C, Tasquier R, Deregibus M, Camussi G, Pascucci L, Marini M, Corradetti B, Bizzarro D, De Vita B *et al.* 2016 Equine Amniotic Microvesicles and Their Anti-Inflammatory Potential in a Tenocyte Model In Vitro. *Stem Cells Development* **25** 610–621.

Li G, Zhang X, Wang H, Wang X, Meng C, Chan C, Yew D, Tsang K, Li K, Tsai S *et al.* 2009 Comparative proteomic analysis of mesenchymal stem cells derived from human bone marrow, umbilical cord and placenta: implication in the migration. *Proteomics* **9** 20–30.

Liang CC, Park AY & Guan JL 2007 In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols* **2** 329–333.

Liang X, Ding Y, Zhang Y, Tse H & Lian Q 2014 Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplantation* **23** 1045–1059.

Maia L, Landim-Alvarenga FC, Da Mota LS, De Assis Golim M, Laufer-Amorim R, De Vita B, Barberini DJ, Listoni AJ, De Moraes CN, Heckler MC & Amorim RM 2013 Immunophenotypic, immunocytochemistry, ultrastructural, and cytogenetic characterization of mesenchymal stem cells from equine bone marrow. *Microsc Res Tech* **76** 618-624.

Mizuno H & Hyakusoku H 2003 Mesengenic potential and future clinical perspective of human processed lipoaspirate cells. *Journal of Nippon Medical School* **70** 300–306.

Mohanty N, Gulati B, Kumar R, Gera S, Kumar P, Somasundaram R & Kumar S 2014 Immunophenotypic characterization and tenogenic differentiation of mesenchymal stromal

514 cells isolated from equine umbilical cord blood. *In Vitro Cellular & Developmental Biology -*

515 *Animal* **50** 538–548.

516 **Nielsen J & McNagny K** 2008 Novel functions of the CD34 family. *Journal of Cell Science*

517 **121** 3683–3692.

518 **Paris DB & Stout TA** 2010 Equine embryos and embryonic stem cells: defining reliable

519 markers of pluripotency. *Theriogenology* **74** 516–524.

520 **Pascucci L, Mercati F, Marini C, Ceccarelli P, Dall'Aglio C, Pedini V & Gargiulo AM**

521 2010 Ultrastructural morphology of equine adipose-derived mesenchymal stem cells. *Histol*

522 *Histopathol* **25** 1277-1285.

523 **Pascucci L, Alessandri G, Dall'Aglio C, Mercati F, Cloliolo P, Bazzucchi C, Dante S,**

524 **Petrini S, Curina G & Ceccarelli P** 2014 Membrane vesicles mediate pro-angiogenic

525 activity of equine adipose-derived mesenchymal stromal cells. *The Veterinary Journal* **202**

526 361–366.

527 **Pascucci L, Dall'Aglio C, Bazzucchi C, Mercati F, Mancini M, Pessina A, Alessandri G,**

528 **Giammaroli M, Dante S, Brunati G** *et al.* 2015 Horse adipose-derived mesenchymal

529 stromal cells constitutively produce membrane vesicles: a morphological study. **30** 549–557.

530 **Pasquinelli G, Tazzari PL, Ricci F, Vaselli C, Buzzi M, Conte R, Orrico C, Foroni L,**

531 **Stella A, Alviano F** *et al.* 2007 Ultrastructural characteristics of human mesenchymal stromal

532 (stem) cells derived from bone marrow and term placenta. *Ultrastructural Pathology* **31** 23–

533 31.

534 **Pera MF** 2009 Stem cells: Low-risk reprogramming. *Nature* **458** 715–716.

535 **Perrini C, Strillacci MG, Bagnato A, Esposti P, Marini MG, Corradetti B, Bizzaro D,**

536 **Idda A, Ledda S, Capra E, Pizzi F, Lange-Consiglio A & Cremonesi F** (2016)

537 Microvesicles secreted from equine amniotic-derived cells and their potential role in reducing

538 inflammation in endometrial cells in an in-vitro model. *Stem Cell Research and Therapy* **18**
539 169.

540 **Rainaldi G, Pinto B, Piras A, Vatteroni L, Simi S & Citti L** 1991 Reduction of
541 proliferative heterogeneity of CHEF18 Chinese hamster cell line during the progression
542 toward tumorigenicity. *In Vitro Cellular & Developmental Biology 27A* **27** 949–952.

543 **Rossi B, Merlo B, Colleoni S, Iacono E, Tazzari PL, Ricci F, Lazzari G & Galli C** 2014
544 Isolation and in vitro characterization of bovine amniotic fluid derived stem cells at different
545 trimesters of pregnancy. *Stem Cell Reviews* **10** 712–724.

546 **Saadeldin IM, Oh HJ & Lee BC** 2015 Embryonic-maternal cross-talk via exosomes:
547 potential implications. *Stem Cells Cloning* **8** 103–107.

548 **Sidney LE, Branch MJ, Dunphy SE, Dua HS & Hopkinson A** 2014 Concise Review:
549 Evidence for CD34 as a Common Marker for Diverse Progenitors. *Stem Cells (Dayton, Ohio)*
550 **32** 1380–1389.

551 **Subramanian A, Fong C, Biswas A & Bongso A** 2015 Comparative Characterization of
552 Cells from the Various Compartments of the Human Umbilical Cord Shows that the
553 Wharton's Jelly Compartment Provides the Best Source of Clinically Utilizable Mesenchymal
554 Stem Cells. *Plos One* **10** e0127992.

555 **Visser M & Pollitt C** 2011 Lamellar leukocyte infiltration and involvement of IL-6 during
556 oligofructose-induced equine laminitis development. *Veterinary Immunology and*
557 *Immunopathology* **144** 120–128.

558 **Wang W, Itaka K, Ohba S, Nishiyama N, Chung U, Yamasaki Y & Kataoka K** 2009 3D
559 spheroid culture system on micropatterned substrates for improved differentiation efficiency
560 of multipotent mesenchymal stem cells. *Biomaterials* **30** 2705–2715.

561 **Yagi H, Soto-Gutierrez A, Parekkadan B, Kitagawa Y, Tompkins RG, Kobayashi N &**

562 **Yarmush ML** 2010 Mesenchymal stem cells: mechanisms of immunomodulation and

563 homing. *Cell Transplant* **19** 667–679.

564 **Yazdanpanah G, Paeini-Vayghan G, Asadi S & Niknejad H** 2015 The effects of

565 cryopreservation on angiogenesis modulation activity of human amniotic membrane.

566 *Cryobiology* **71** 413–418.

567