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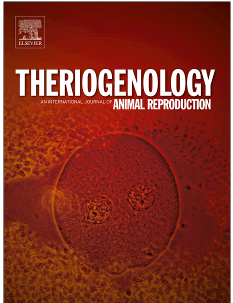

Isolation, molecular characterization and *in vitro* differentiation of bovine Wharton's jelly-derived multipotent mesenchymal cells

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1 **REVISED**

2 **Isolation, molecular characterization and *in vitro* differentiation of bovine Wharton's jelly-**
3 **derived multipotent mesenchymal cells**

4

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13

14 **ABSTRACT**

15 Extra-fetal tissues are a non-controversial and inexhaustible source of mesenchymal stem cells
16 (MSCs) that can be harvested non-invasively at low cost. In the veterinary field, as in man, stem
17 cells derived from extra-fetal tissues express plasticity, reduced immunogenicity, and have high
18 anti-inflammatory potential making them promising candidates for treatment of many diseases.
19 Umbilical cord mesenchymal cells have been isolated and characterized in different species, and
20 have recently been investigated as potential candidates in regenerative medicine. In this study, cells
21 derived from bovine Wharton's jelly (WJ) were isolated for the first time by enzymatic methods,
22 frozen/thawed, cultivated for at least ten passages and characterized. Wharton's jelly (WJ)-derived
23 cells readily attached to plastic culture dishes displaying typical fibroblast-like morphology and,
24 although their proliferative capacity decreased to the seventh passage, these cells showed a mean
25 doubling time of 34.55 ± 6.33 hours and a mean frequency of 1 colony forming unit fibroblast-like
26 (CFU-F) for every 221.68 plated cells. The results of molecular biology studies and flow cytometry

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analyses revealed that WJ-derived cells showed the typical antigen profile of MSCs and were positive for CD29, CD44, CD105, CD166, Oct-4 and c-Myc. They were negative for CD34 and CD14. Remarkably, WJ-derived cells showed differentiation ability. After culture in induced media, WJ-derived cells were able to differentiate into osteogenic, adipogenic, chondrogenic and neurogenic lines as shown by positive staining and expression of specific markers. On PCR analysis, these cells were negative for *MHC-II* and positive for *MHC-I*, thus reinforcing the role of extra-fetal tissue as an allogenic source for bovine cell-based therapies. These results provide evidence that bovine WJ-derived cells may have the potential to differentiate to repair damaged tissues and reinforce the importance of extra-fetal tissues as stem cell sources in veterinary regenerative medicine. A more detailed evaluation of their immunological properties is necessary to better understand their potential role in cellular therapy.

Key words: bovine, Wharton's jelly, stromal cells, characterization

1. INTRODUCTION

Increasing interest in veterinary stem cell therapy has led to research into new stem cell sources that can supply sufficient numbers of cells whilst minimizing risks to donors and recipients [1]. Extra-fetal stem cells are being investigated for this purpose in large animals [2]. Extra-fetal stem cells have been isolated from umbilical cord blood, amniotic fluid, amniotic membrane, umbilical cord matrix, yolk sac and placenta [3-9]. It has been shown that they possess properties of mesenchymal stem cells, and their application in regenerative medicine has increased over the past few years [10]. These studies show that umbilical cord, previously considered as a biomedical waste, is a source of stem cells with promising therapeutic applications in man as well as in livestock species.

The umbilical cord provides an inexhaustible source of stem cells and presents few, if any, ethical concerns. Indeed, umbilical cord can be collected after parturition since it is considered a waste tissue. In addition, the process of obtaining the cord tissue is relatively simple and non- invasive.

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53 Mesenchymal stem cells (MSCs) have been isolated from different compartments of the umbilical
54 cord in different species. In particular, they were obtained invasively, by a surgical intrauterine
55 approach, from umbilical cord blood of sheep [11,12], non-invasively at the time of birth in the
56 horse [13], in cattle, after caesarean section delivery [14], and from canine and feline fetuses at birth
57 [15,16]. In 2006, for the first time in veterinary medicine, MSCs from cordon matrix, called
58 Wharton's jelly (WJ), were obtained from porcine umbilical cord [17]. Wharton's jelly is the
59 embryonic mucous connective tissue lying between the amniotic epithelium and the umbilical
60 vessels [18]. It encloses the yolk sac, which is the source of primordial germ cells and the first
61 hematopoietic stem cells [18]. Extra-embryonic tissues, which originate from the hypoblast and the
62 trophectoderm, do not participate in gastrulation, therefore they are less differentiated than adult
63 somatic tissues such as bone marrow or adipose tissue [2]. Wharton's jelly is a rich source of MSCs
64 [19-21]. Recently, primitive MSCs were isolated from the umbilical cord matrix in caprine [22,23],
65 canine [24] and equine species [4,13,25]. These cells show specific markers of pluripotency and
66 MSCs markers and are able to differentiate into adipogenic, chondrogenic, osteogenic and
67 neurogenic tissues [2]. The similarities in growth kinetics and expression of markers of pluripotency
68 indicate their close resemblance to embryonic stem cells [26]. These markers, found in mouse and
69 human embryonic stem cells, include the POU (Pit/Oct/Unc) domain-containing protein Oct-4, Sox-
70 2 and Nanog. Some authors have shown that human umbilical cord matrix expresses low levels of
71 transcription factors that play a central role in the regulation of pluripotency and self-renewal [18].
72 In contrast however, Carlin et al. [17] showed that cells derived from porcine umbilical cord matrix
73 express markers of embryonic lineage Oct-3/4, Sox2 and Nanog. Contrary to observations in adult
74 MSCs, WJ-MSCs share properties unique to fetal-derived MSCs, such as more rapid proliferation
75 and greater *ex vivo* expansion capabilities [18,27]. Moreover, they have high potential to be
76 differentiated *in vitro* [28], they express HLA-class I surface markers but do not express HLA-class
77 II markers [29], and they are immunosuppressive in mixed lymphocyte assays by inhibition of T-
78 cell proliferation [30,31]. For these reasons, these cells have raised interest for their potential uses in

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79 cell and gene therapy, cloning, virological and biotechnological studies [32].

80 Despite the importance of bovine species as models for *in vivo* studies, little it is known about
81 bovine MSCs. So far, they have been derived from umbilical cord blood [14], bone marrow [33,34]
82 and amniotic membrane or amniotic fluid [8]. To date, only one paper on isolation and
83 characterization of MSCs from bovine umbilical cord matrix [32] had been published but these cells
84 were isolated by migration techniques and not by classical enzymatic digestion. The mechanical and
85 enzymatic disaggregation of the tissue avoids problems of selection by migration, but perhaps more
86 importantly, yields a higher number of cells more representative of the whole tissue in a shorter
87 time. However, as well as the primary explant technique selecting on the basis of cell migration, the
88 dissociation technique selects cells resistant to disaggregation but still capable of attachment [35].

89 The isolation of bovine MSCs from fetal adnexa is an interesting prospect because of the potential
90 for these cells to be used for biotechnological applications. For the first time, we isolated, by
91 enzymatic methods, presumptive MSCs from bovine WJ and were able to characterize them in
92 terms of morphology, specific mesenchymal or pluripotent markers, proliferative and differentiation
93 potential.

94

95 2. MATERIALS AND METHODS

96 *2.1 Materials*

97 Chemicals were obtained from Sigma Chemical (Milan, Italy) and tissue culture plastic dishes from
98 Euroclone (Milan, Italy) unless otherwise specified.

99

100 *2.2 Tissue collection*

101 This study was approved by the Ethical Committee of the University of Milan and written owner
102 consent was given. All procedures were conducted following standard veterinary practice and in
103 accordance with 2010/63 EU directive on animal protection and Italian Law (D.L. No. 116/1992).
104 Fresh bovine umbilical cords were obtained after full-term births.

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105 Bovine umbilical cords (n=3) were obtained from three cows following normal term pregnancies
106 with spontaneous parturition in accordance with veterinary practice standard. Before the cows stood
107 up breaking the cord, a surgical tape was placed at the calf junction and a second tie was tightened
108 at approximately 30/40 cm from the first. The tie-limited cord portion was cut away with scissors.
109 The harvested segment of the cord was washed twice in sterile saline solution and kept at 4°C in
110 saline solution supplemented with 4 μg/mL amphotericin, 100 UI/mL penicillin and 100 μg/mL
111 streptomycin and processed within 12 hours of collection.

112

113 *2.3 Isolation and culture of WJ-derived cells*

114 At the laboratory, the loose amnion was removed from the exterior of the cords and the cords were
115 incised longitudinally to expose and remove umbilical vessels (arteries and veins). The remaining
116 WJ-containing tissue was minced into small pieces using scissors. The tissue was digested in HG-
117 DMEM supplemented with 1mg/mL collagenase type I at 38.5 °C for 8 hours. After incubation,
118 collagenase was inactivated by diluting 1:1 with HG-DMEM supplemented with 10% fetal calf
119 serum (FCS). The digested suspension was filtered on an 80 μm strainer, centrifuged at 300 *g* for 10
120 minutes and washed twice in PBS. Before seeding, cells were counted using a Burker chamber with
121 the Trypan Blue dye exclusion assay. WJ-cell cultures were established in HG-DMEM standard
122 medium composed of 10% FCS, penicillin (100 UI/mL)-streptomycin (100 μg/mL), 0.25 μg/mL
123 amphotericin B, 2 mM L-glutamine and 10 ng/mL EGF. Cultures were established at a density of
124 $1x10^5$ cells/cm^2 in T75 culture flasks. The flasks were incubated at 38.5 °C with 5% CO_2 and 90%
125 humidity. The medium was replaced after 72 hours to remove non-adherent cells and then replaced
126 twice weekly until cells reached approximately 80% confluence. Cells were then detached with
127 0.05% trypsin-EDTA, counted, and redistributed into new culture flasks at a density of $1x10^4$
128 cells/cm^2 to maintain and expand the culture for ten passages (P).

129

130

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131 *2.4 Cryopreservation and thawing*

132 Cells at P3 were cryopreserved. Briefly, the confluent cultures were treated with 0.05% trypsin-
133 EDTA and washed by centrifugation (200*g* at 4 °C, for 5 minutes) with cell culture medium
134 supplemented with 10% FCS to neutralize trypsin-EDTA. The cell pellet obtained was resuspended
135 in pre-cooled (4 °C) cryopreservation media in 1-mL cryovials. The cryopreservation medium was
136 90% (v/v) FBS and 10% dimethyl sulfoxide. The cryovials were maintained at -80 °C overnight and
137 then plunged into liquid nitrogen (-196 °C). After a minimum of one month of cryopreservation, the
138 cells were thawed in a water bath at 37 °C. The cells were diluted in culture medium and
139 centrifuged twice at 200*g* for 10 minutes. The cell pellet was resuspended in culture medium and
140 plated in T25 culture flasks. Aliquots of these cells were kept to evaluate the cell viability using
141 Burker hemocytometer chamber using the trypan blue dye exclusion method, under phase contrast
142 microscopy (Nikon Eclipse Ti, Tokyo, Japan). After thawing, other aliquots were used for
143 population doubling studies, or expanded until P3 to evaluate specific MSC marker expression and
144 multipotent differentiation capacity in comparison to freshly isolated cells.

145

146 *2.5 Proliferation rate and CFU-assay*

147 Proliferation of MSCs was determined as previously reported [6]. Doubling time (DT) from P 1-10
148 was assessed by plating $9x10^3$ cells into six-well tissue culture plates. Every 4 days, cells were
149 trypsinized, counted and reseeded at the same density. Mean DT was calculated from day 0 to day
150 4. The DT value was obtained for each P according to the formula DT=CT/CD, where CT
151 represents the culture time and CD=log(N_c/N_o)/log2 represents the number of cell generations (N_c
152 represents the number of cells at confluence, N_o represents the number of seeded cells). Data
153 representative of three independent experiments are reported.

154 To obtain the cell proliferation growth curve, cells at P0, P3 and P5 were seeded into six-well tissue
155 culture dishes at a density of 1×10^3 cells/cm^2. Every 2 days, until 14 days of culture, one well out

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156 of the six was trypsinized and cells were counted using the Trypan blue dye exclusion method with
157 a Burker chamber.
158 Colony-forming unit (CFU) assays were performed at P0 on freshly isolated cells at different
159 densities (100, 250, 500 and 1000 cells/cm^2). Cells were plated in six-well plates for two weeks in
160 HG-DMEM standard medium. Then, colonies were fixed for 1 hour with 4% formalin and stained
161 with 1% methylene blue for 15 minutes in 10 mM borate buffer at room temperature. Colonies
162 formed by 16 to 20 nucleated cells were counted under a BX71 microscope (Olympus Italia, Srl,
163 Milano, Italy).
164
165 *2.6 Osteogenic, adipogenic, chondrogenic and neurogenic cell differentiation*
166 All the differentiation tests were performed when cells reached P3.
167 For osteogenic differentiation, cells were placed in plastic six-well plates at a density of 28 x10^3
168 cells per well. After the cells had adhered to the plastic, the inducer medium was added to the plate
169 for 21 days and refreshed every three days. The medium was composed of HG-DMEM, 10% FCS,
170 penicillin (100 UI/mL)-streptomycin (100 μg/mL), 0.25 μg/mL amphotericin B, 200 mM L-
171 glutamine, 0.25 mM ascorbic acid, 10 mM β-Glycerophosphate, and 0.1 uM Dexamethasone [36].
172 Osteogenic differentiation was confirmed by positive staining of the extracellular calcium matrix
173 using Von Kossa staining.
174 For adipogenic differentiation, cells were placed in plastic six-well plates at a density of 28 x10^3
175 cells per well. After the cells had adhered to the plastic, the inducer medium and the maintaining
176 medium were added alternately, every 3 days for a total of 21 days. The inducer medium was
177 composed of HG-DMEM, 10% FCS, penicillin (100 UI/mL)-streptomycin (100 μg/mL), 0.25
178 μg/mL amphotericin B, 200 mM L-glutamine, 0.1% insulin, 0.1 uM dexamethasone, and 1%
179 indomethacin. The maintaining medium was composed of HG-DMEM, 10% FCS, penicillin (100
180 UI/mL)-streptomycin (100 μg/mL), 0.25 μg/mL amphotericin B, and 0.1% insulin [36]. Adipogenic
181 differentiation was confirmed by positive staining of the lipid structures using Oil Red O staining.

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182 For chondrogenic differentiation, cells were cultured in DMEM low-glucose, containing 100U/mL
183 penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, 2 mM/l L-glutamine, 100 nM
184 dexamethasone, 50 µg/mL L-ascorbic acid 2-phosphate, 1 mM sodium pyruvate (BDH, Atlanta,
185 USA), 40 µg/mL proline, ITS (insulin 5 µg/mL, transferrin 5 µg/mL, selenous acid 5 ng/mL) and 5
186 ng/mL transforming growth factor-β3 (Peprovet, DBA Italia, 100-36E). Chondrogenic
187 differentiation was assessed after incubating cells for up to 3 weeks [37]. Differentiation was
188 evaluated by Alcian blue staining.
189 For neurogenic differentiation, cells were placed in plastic six-well plates at a density of 28 $x10^3$
190 cells per well. After the cells had adhered to the plastic, the pre-inducer medium was administrated
191 to the plates for 1 day, followed by administration of inducer medium for 7 days. The pre-inducer
192 medium was composed of HG-DMEM, 20% FCS, penicillin (100 UI/mL)-streptomycin (100
193 µg/mL), 0.25 µg/mL amphotericin B, and 0.0007% β-mercaptoethanol [15,38]. The inducer
194 medium was composed of HG-DMEM, 2% FCS, penicillin (100 UI/mL)-streptomycin (100
195 µg/mL), 0.25 µg/mL amphotericin B, 1% DMSO, and 0.36 mg/mL BHA [39]. Neurogenic
196 differentiation was confirmed by positive staining of the Nissl substance and granules, using Nissl
197 staining.
198 For each differentiation experiment, a control group was performed by seeding cells at lower
199 density ($9.5x10^3$) and feeding with HG-DMEM standard medium. These cells were stained using
200 the same protocol as the treated cells. At the end of the differentiations, aliquots of non-stained cells
201 were harvested and stored at -80 °C for further molecular analysis.
202

203 *2.7 RNA extraction and Reverse Transcription-PCR analysis*

204 Expression of specific MSC (*CD29*, *CD44*, *CD105*, *CD166*), pluripotent- (*Oct-4* and *c-Myc*) and
205 hematopoietic (*CD34, CD14, CD45*) markers was investigated by RT-PCR analysis on fresh and
206 thawed undifferentiated cells at P3. To evaluate whether cells could be well tolerated by the host
207 once transplanted, expression of the Major Histocompatibility Complex, class I (*MHC-I*) and II

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208 (*MHC-II*) was assessed. Total RNA was extracted from bovine WJ-derived cells using Trizol
209 reagent (Invitrogen, Monza, Italy), followed by DNase treatment according to the manufacturer's
210 specifications. RNA concentration and purity were measured using a NanoDrop ND1000
211 spectrophotometer (*NanoDrop* Technologies, Wilmington, DE, USA). cDNA was synthesized from
212 500 ng total RNA, using the iScript retrotranscription kit (Bio-Rad Laboratories, Hercules, CA,
213 USA). Conventional PCR was performed in a 25 mL final volume with DreamTaq DNA
214 Polymerase (Fermentas, St. Leon Rot, Germany) under the following conditions: initial
215 denaturation at 95 °C for 2 minutes, 32 cycles at 95 °C for 30 seconds (denaturation), 55–63 °C for
216 30 seconds (annealing), 72 °C for 30 seconds (elongation) and final elongation at 72 °C for 10
217 minutes.
218 For differentiation experiments, total RNA was extracted from undifferentiated (control cells) and
219 from induced WJ-derived cells and RT-PCR analysis was performed as described above. Bovine
220 adult tissues (bone, fat, cartilage and spinal cord) were employed as positive controls for assessing
221 the expression of *BGLAP, SPP1* and *SPARC* for osteogenic differentiation, peroxisome proliferator-
222 activated receptor-gamma (*PPAR-y*), and leptin (*LEP*) for adipogenesis, collagen type 2α1
223 (*COL2A1*) and aggrecan (*ACAN*) for chondrogenesis, and glial fibrillary acidic protein (*GFAP*) and
224 nestin (*NES*) for neurogenesis. Bovine-specific oligonucleotide primers were designed using open
225 source PerlPrimer software v. 1.1.17, based on available NCBI *Bos taurus* sequences or on Mammal
226 multi-aligned sequences. Primers were designed across an exon–exon junction in order to avoid
227 DNA amplification. Primers were used at 300 nM final concentrations. Their sequences and the
228 conditions used to amplify each gene are shown in Table 2. *GAPDH* was employed as a reference
229 gene.
230
231 *2.8 Flow cytometry*
232 WJ-derived cells were analyzed by flow cytometry to determine the percentage of mesenchymal-
233 (CD105, CD166), hematopoietic (CD34) and immunogenic (MHC-II) markers after isolation (P0).

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234 For CD105, CD166 and CD34, primary mouse monoclonal antibodies and secondary antibodies
235 rabbit anti-mouse FITC (Sigma, Milan, Italy) were used. For MHC-II, primary rat monoclonal
236 antibody and secondary rabbit anti-rat FITC (Sigma) were used. Staining was performed as
237 previously reported [40]. Cells ($1x10^6$ cells/mL) were labeled with primary antibodies in PBS with
238 3% of bovine serum albumin (BSA) (BDH; VWR International Ltd, Poole, UK) for 45 minutes at
239 room temperature in the dark, followed by washing in cold PBS and a final incubation with
240 secondary antibodies (1:50) for 30 minutes at room temperature in the dark. After incubation, cells
241 were washed twice in ice-cold PBS and analyzed using a Millipore Guava easyCyte Single Sample
242 Flow Cytometer. A minimum of 10,000 cells was acquired for each sample and analyzed in the FL1
243 channel.
244 The negative pattern was examined by applying the same cell suspension with the first incubation,
245 and the result was included on the global compensation, in order to exclude auto fluorescence. A
246 488 nm filter was used in each analysis.
247 Off-line analyses of the flow cytometry standard (FCS) files were performed using Weasel software
248 v.2.5 (http://en.bio-soft.net/other/WEASEL.htmL).
249
250 *2.9 Statistical analysis*
251 Statistical analysis was performed using GraphPad Instat 3.00 for Windows (GraphPad Software,
252 La Jolla, CA, USA). Three replicates were performed for each experiment (DT and CFU) and the
253 results reported as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) for
254 multiple comparisons by Student-Newman-Keuls multiple comparison tests was used. $P<0.05$ was
255 considered as significant.
256
257 **3. RESULTS**
258 *3.1 Cell morphology*
259 The cellular yield was approximately $3x10^6$ cells per gram of minced WJ. The initial viability was

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260 greater than 95%.

261 Cells were selected purely on their ability to adhere to plastic. Isolated cells readily attached to
262 plastic culture dishes. Colonies started to appear within the first two days, reaching confluence after
263 5 days. WJ-derived cells were a morphologically homogeneous population of fibroblast-like cells in
264 all passages of cell culture (Figure 1 A).

265 After thawing (at P3), viability was 80%. WJ-derived cells conserved their fibroblast-like shape.

266

267 *3.2 Proliferation studies* Cells WJ-derived cells were able to proliferate, reaching confluence in up
268 to 10 passages.

269 At P1, the growth curve showed an initial lag phase of 3-4 days longer than that seen in the other
270 passages and a subsequent log phase until 14 days. The WJ-derived cells showed more extensive
271 proliferation at P5 but the intensity of proliferation was similar in all passages in the final culture
272 days (Fig 1B).

273 DT remained constant until the seventh passage, then decreased significantly ($P<0.05$) until passage
274 10. The mean DT was 1.44 ± 0.24 days or 34.55 ± 6.33 hours (Fig 1C).

275 After thawing, the mean DT was 1.59 ± 0.06 days or 38.21 ± 1.68 hours (Fig 1C) with no statistical
276 difference compared to fresh cells.

277 The number of cell colonies formed was counted at P0 on fresh cells. These cells were able to form
278 an average of 1 CFU-F) (Fig 1D) for 221.68 ± 3.86 seeded cells. The highest number of colonies
279 was found at the greatest density of seeding (Table 1).

280

281 *3.3 Molecular characterization*

282 As shown by RT-PCR, WJ-derived cells expressed MSC-markers (*CD29*, *CD44, CD105, CD166*)
283 and lacked hematopoietic ones (*CD34* and *CD14*) from P0 to P10. *MHC-I* expression was present
284 while *MHC-II* was not. Moreover, undifferentiated MSCs were found to express *Oct-4* and *c-Myc,*
285 essential transcription factors for maintaining the primitive pluripotent state of embryonic stem

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286 cells. After thawing, cells studied at P3 expressed the same MSC-mRNA markers such as *CD29,*
287 *CD44, CD105, CD166, Oct-4*, *c-Myc* and *MHC-I*, as freshly isolated cells, but not *CD34, CD14* and
288 *MHC-II*. (Figure 2A).
289
290 *3.4 Flow cytometry analysis*
291 Flow cytometry was used to evaluate the homogeneity of the cell population and to identify the
292 subset of mesenchymal, hematopoietic and immunogenic cells. The cell populations tested were all
293 $CD105^+$ and $CD166^+$, but negative for CD34 and MHC-II, as shown in Fig 2B.
294
295 *3.5 Differentiation assay*
296 The results of all differentiation assays are shown in Fig 3.
297 *Osteogenic differentiation potential*. After 21 days of induction, osteogenic differentiation was
298 confirmed by von Kossa staining. The control (non-induced cells) was negative for von Kossa
299 staining. RT-PCR analysis of *SPP1* and *SPARC* mRNA expression confirmed osteogenic induction
300 but *BGLAP* was not expressed in induced cells.
301 *Adipogenic differentiation potential*. Cells were able to undergo adipogenic differentiation, as
302 demonstrated by the development of positive staining for Oil Red O after 3 weeks of culture in
303 adipogenic induction medium. Control cells, maintained in regular control medium, showed no lipid
304 deposits. RT-PCR analysis of *PPAR-y* and *LEP* mRNA expression confirmed adipogenic induction.
305 *Chondrogenic differentiation potential.* Differentiation was identified by marked deposition of
306 glycosaminoglycans in the matrix, stained with Alcian blue. The presence of COL2A1 and ACAN
307 mRNA confirmed chondrogenic induction for this cell population.
308 *Neurogenic differentiation potential*. After 3 days of induction, neurogenic differentiation was
309 confirmed by the morphology of the cells. The WJ-derived cells adopted the typical morphology of
310 neural cells with dendrite-like processes. The presence of *GFAP* mRNA suggested that under these
311 culture conditions, WJ-derived cells were induced to differentiate into glial cells. RT-PCR analysis

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312 of bovine adult tissues (bone, fat, cartilage and spinal cord) showed expression of the specific
313 studied genes.
314 The frozen-thawed cells were able to differentiate toward the same lineages as freshly isolated cells
315 (data not shown).
316
317 **4. DISCUSSION**
318 This work allowed the isolation, characterization and differentiation of bovine stem cells derived
319 from WJ. Our findings suggest that this tissue is a reliable source of presumptive stem cells,
320 displaying intermediate features between adult and embryonic stem cells. These cells havewide
321 potential clinical applications, because of their low immunogenicity and high differentiation
322 potential. After digestion, large numbers of WJ-derived cells with > 95% viability (optimal value in
323 terms of plating efficiency and cellular growth) were obtained. When cultured, these cells
324 demonstrated strong adherence to plastic dishes and developed fibroblast-like morphology over
325 time. Adherence is a fundamental property for the culture of stem cells [41]. The proliferation
326 studies showed that WJ-derived cells reached high plating efficiency and had a high proliferation
327 rate *in vitro* until P10, demonstrating a growth curve with a *lag* phase of few hours and an intensive
328 *log* phase of 12 days. Moreover, the mean value of DT for 10 passages was 34.55 hours. During this
329 intensive proliferation, the cells maintained their morphological characteristics. These data are in
330 agreement with those obtained by other researchers who reported a high proliferation rate of human
331 [37,42], equine [6], bovine [8] and feline [43] extra-fetal derived cells. It is very difficult to
332 compare these data with those obtained from Cardoso et al. [32] because this study only reported
333 the number of cells per mL found at different passages and the DT value was not calculated.
334 Bovine WJ-derived cells also showed the ability to produce clones. When seeded at different
335 densities, they were able to form clones with a frequency that increased with the cell-seeding
336 density, suggesting paracrine signaling between cells at P0 [44]. Moreover, WJ-derived cells
337 showed a typical expression pattern expected for cultured stem cells [41] when analyzed by RT-

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338 PCR. Indeed, these cells expressed a pattern of mesenchymal (*CD29*, *CD44, CD105, CD73*,
339 *CD166*) and pluripotency (*Oct4*, *c-Myc*) genes with no expression of the hematopoietic *CD34*. The
340 pluripotency genes are essential transcription factors for maintaining the primitive pluripotent state
341 of embryonic stem cells. These data confirm the results obtained in equine, canine and bovine WJ
342 [6,8,24,32,45-47] where pluripotent- and mesenchymal-associated markers were expressed.
343 For the first time, the expression of *MHC-I* and *MHC-II,* related to cell immunogenicity, was also
344 evaluated to assess the usefulness of bovine WJ-derived cells for cell therapy. At each passage,
345 these cells were negative for *MHC-II* and positive for *MHC-I*, consistent with findings of previous
346 publications [6,8]. These findings reinforce the role of the extra-fetal tissue as an allogenic source
347 for cell-based therapies in cattle. It is important to underline that RT-PCR alone is not useful for
348 characterizing WJ-derived cells and that quantitative analyses are needed to make meaningful
349 statements about their gene expression. Flow cytometry provides useful quantitative data on the
350 percentage of cell reactivity. Indeed, we showed that >90% cells were positive for CD105 and
351 CD73 while < 10% were positive for CD34 and MHC-II. These data confirm the mesenchymal
352 nature of isolated cells, the lack of immunogenicity and underline the homogeneity of this cell line.
353 The capacity of MSCs to differentiate into a variety of cell types (adipocytes, osteocytes and
354 chondrocytes) [48,49], has aroused interest in cell and gene therapy. Bovine WJ-derived cells,
355 obtained by enzymatic digestion, were able to differentiate into osteocytes, adipocytes,
356 chondrocytes and neuron-like cells in the same way as cells obtained by non-enzymatic digestion
357 [32]. This suggests that these cells are capable of differentiation into multiple germ layers, an
358 essential characteristic also observed in the pig [17], dog [24], horse [4] and chicken [50]. After 21
359 days of induction, mineral deposits were confirmed by Von Kossa staining and by the expression of
360 *SPP1* and *SPARC* but not of *BGLAP*. This might be because *BGLAP* is expressed in terminally
361 differentiated osteoblasts [51]. When stimulated to differentiate towards the adipogenic lineage,
362 bovine WJ-derived cells were positive for Oil Red O staining and expressed genes involved in lipid
363 biosynthesis and storage. mRNAs for *PPAR-γ*, crucial for the preadipocyte commitment [52], and

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364 *LEP*, regarded as a late marker of adipocyte differentiation, were detected. The potential of bovine
365 WJ-derived cells to undergo chondrogenesis was confirmed by positive Alcian blue staining and
366 identification of markers commonly associated with the chondrocyte phenotype such as collagen
367 type II and aggrecan, the most essential cartilage proteoglycan and key markers of chondrocyte
368 differentiation [53]. *ACAN* expression was demonstrated, whereas a basal level of *COL2A1* was
369 detected. The low expression of *COL2A1* might be related to the culture conditions in this study
370 since chondrogenic differentiation of MSCs in monolayer culture appears to be dose-dependent and
371 time-dependent in relation to the bioactive factors used [32,33]. *GFAP* and nestin, markers,
372 expressed in neuronal precursor stem cells, have been detected in WJ-derived cells. The expression
373 of both markers is probably related to the ability of these cells to differentiate either towards the
374 glial cell lineage, as previously shown by Miki et al. [54] for 95% of cells isolated from human
375 amnion, or toward neurogenic line as previously observed in pigs [55].
376 Whatever the reason, this cell line converted into a typical neuron-like morphology when
377 appropriately induced.
378 Our findings suggest that, in agreement with reports of other researchers in several species [6,45-
379 47,56], bovine WJ represents an alternative source of progenitor cells, that can be obtained by
380 enzymatic methods for use in cell-based therapies. In our study, the digestion of tissue did not result
381 in a reduced cellular viability or degradation of cellular surface receptors or alteration of cellular
382 function as reported by Jeschke et al. [57].
383 Moreover, after thawing, the cryopreserved cells had a high level of viability (80%) and could be
384 successfully expanded and differentiated. This demonstrates that bovine umbilical cord matrix cells
385 can tolerate freezing without loss of functional integrity in terms of morphology, presence of
386 specific stemness markers and differentiation potential, although renewal capacity was slightly
387 lower than that observed in freshly isolated cells.
388 In conclusion, these data confirm that bovine WJ contain a niche of MSCs. However, further
389 investigation, including pre-clinical studies and further study of immunological properties, are

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390 needed to better understand their role in cellular therapy before *in vivo* applications of WJ-derived
391 cells are considered. To date, there is only a single literature report of transplantation of caprine WJ-
392 derived cells for wound healing, which showed promising effects [23]. The findings of this study
393 reinforce the emerging importance of extra-embryonic tissues for derivation of cells that may be
394 ideal tools in veterinary regenerative medicine.

395

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400

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617

618 **DECLARATION OF INTEREST**

619 The authors declare that there are no conflicts of interest that could be perceived as prejudicing the
620 impartiality of the reported research.

621

622 **CONTRIBUTORS**

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623 ALC designed the study, performed isolation cells, acquired, analyzed and interpreted data, and
624 wrote the article
625 CP performed proliferation study, molecular characterization and approved the final version of
626 manuscript
627 AB performed cytofluorimetric analyses and approved the final version of manuscript
628 PE performed differentiation study and approved the final version of manuscript
629 FC designed the study, analyzed and interpreted data and revised the manuscript
630 LV conceived and designed the study, collected the umbilical cords, analyzed and interpreted data,
631 and wrote the article
632

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633 **Table 1.** CFU assay

634

Density cells/cm^2	Total cells	CFU	1 CFU each
100	950	1.5±0.71^{a}	633.33
250	2375	22.33±5.17^{b}	106.36
500	4750	57.92±4.31^{c}	82.01
1000	9500	146.15±2.78^{c}	65.00

635 Different small letters superscripts (a,b,c) indicate statistically different comparisons (P<0.05) between cell densities in
636 the WJ group.
637

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638 **Table 2.** Oligonucleotide sequences used for RT-PCR analysis.

639

Markers	Forward (5'→3')	Reverse (5'→3')	Annealing T	Product lenght
Integrin β-1 (*CD29*)	GTTGGTTCTGCAGTTACGATCAG	AACCAAACCCAATTCGGAAGTC	52°C	203
CD44 antigen (*CD44*)	AACAGTAGGAGAAGGTGTGG	TCATGAACTGGTCTTGGGTC	61°C	166
Endoglin (*CD105*)	ACAAGTCTTGCAGAAACAGTC	GATGTCTGGAGAGTCAGCTC	61°C	182
ALCAM (*CD166*)	GTATTTATTGCCTTCAGGTCCT	TCTACCAGGGAGCATTTATAGTC	59°C	755
octamer-binding transcription factor 4 (*Oct-4*)	CACACTAGGATATACCCAGGC	GGAGATATGCAAGGCAGAGA	60°C	177
v-myc avian myelocytomatosis viral oncogene homolog (*c-Myc*)	GCGCCGCATTCGCGAAACTT	TGAGGGGCATCGCTGCAAGC	58°C	214
CD34 molecule (*CD34*)	CCTGAAGCTAAATGAGACCT	AACTTTCTGTCCTGTTGGTC	58°C	173
CD14 molecule (*CD14*)	TCCGAAGCCTGACTGGTCTA	TGTCGGCTCCCTTGAGAAAC	56°C	104
Major histocompatibility complex I (*MHC-I*)	GATCTCACTGACCTGGCA	CTGAGGAGGTTCCCATCTC	60°C	199
Major histocompatibility complex II (*MHC-II*)	CCTCGCTTGCCTGAATTTGC	ACAGGTGCCGACTGATGC	53°C	299
Bone Gamma-Carboxyglutamate (Gla) Protein *(BGLAP)*	TCGGGCAAAGGCGCAGCCTTC	GCAGGGCTGCAAGCTCTAGACG	55°C	231
Secreted Phosphoprotein 1 *(SPP1)*	CGCCGATCTAACGTTCAGAGTC	GACTCTCAATCAGATTGGAATGC	55°C	199
secreted protein acidic and rich in cysteine *(SPARC)*	CTGGTCACGCTGTACGAGAG	CGGTGTGAGACAGGTACCCGT	55°C	232
Leptin (*LEP*)	CAATGACATCTCACACACGCAG	CGGCCAGCAGGTGGAGAAG	55°C	212
Peroxisome Proliferator-activated Receptor (*PPAR*-y)	CGCACTGGAATTAGATGACAGC	CACAATCTGTCTGAGGTCTGTC	55°C	199
Collagen type 1, alpha I (*COL1A1*)	CGCGGATTTGTTGCTGCTTGC	AGGTCCCATCAGCCCCATTGGT	55°C	269
Aggrecan (*ACAN*)	CGCTGTCTCGCCAAGTGTATGG	CGGTTCAGGGATGCTGACACTC	60°C	175
Glial Fibrillary Acidic Protein (*GFAP*)	GGCACCTTGAGGCAGAAGCTC	CTCCTGGAGCTCCCGCACCT	60°C	195
Nestin (*NES*)	ACCACTGAGCAGTTCCAGCTGG	TTGCAGGTGTCTGCAGCCGT	55°C	187
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	ATGAGATCAAGAAGGTGGTG	CCAAATTCATTGTCGTACCAG	60°C	190

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649 **Figure Captions**

650 **Figure 1.** Cell characteristics. (A) Monolayer of WJ-derived cells (A); magnification 20X; scale
651 bar= 20 µm. (B) growth curve at passages 1 (P1), 5 (P5) and 10 (P10); (C) doubling times for both
652 fresh and thawed cells. (D) colony of WJ-derived cells for CFU study. Magnification: X 20; scale
653 bar= 20 µm.

654

655 **Figure 2.** (A) RT–PCR analysis of mesenchymal (*CD29*, *CD44*, *CD105* and *CD166*), pluripotent
656 (*Oct-4* and *c-Myc*), hematopoietic (*CD34* and *CD14*) specific gene expression on WJ-derived fresh
657 and thawed cells. Major histocompatibility complex (*MHC*) I and II gene expression is also
658 reported. *GAPDH* was used as the reference gene. (B) Flow cytometry analysis of the expression of
659 mesenchymal (CD105 and CD166), hematopoietic (CD34) and immunogenic (MHCII) markers.
660 Histograms represent relative number of cells vs. fluorescence intensity (FL1). Black histograms
661 indicate background fluorescence intensity of cells labeled with isotype control antibodies only gray
662 histograms show positivity to the studied antibodies.

663

664 **Figure 3.** Staining of differentiated and control (undifferentiated) WJ-derived cells and respective
665 molecular expression. Osteogenic induced cells were evaluated for von Kossa staining and RT–PCR
666 analysis of *SPP1*, *SPARC* and *BGLAP*. Adipogenic induced cells were evaluated for Oil Red O-
667 stained cytoplasmic neutral lipids and RT–PCR of *PPAR-γ* and *LEP*. Chondrogenic induced cells
668 were evaluated for Alcian blue staining and RT–PCR of *COL2A1* and *ACAN*. Neurogenic induced
669 cells were evaluated for Nissl staining and RT–PCR of *NES* and *GFAP*; magnification: X 20; scale
670 bar=20 µm. *GAPDH* was employed as the reference gene. Bone, adipose tissue, cartilage and spinal
671 cord were used as positive controls.

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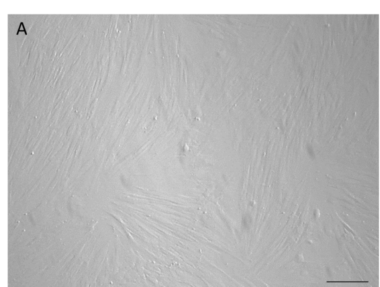

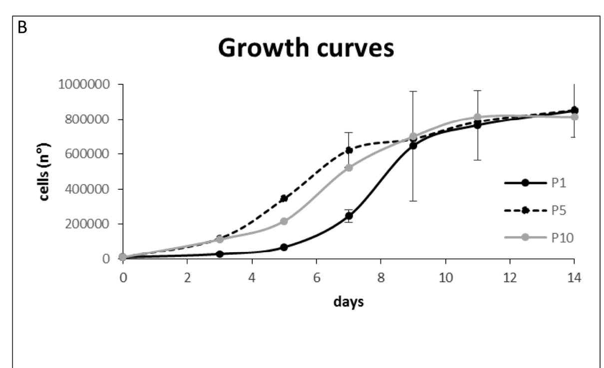

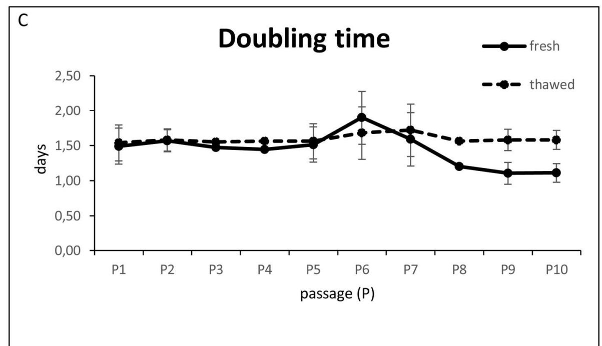

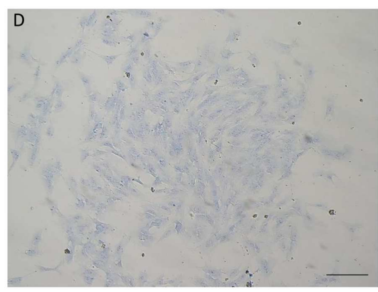

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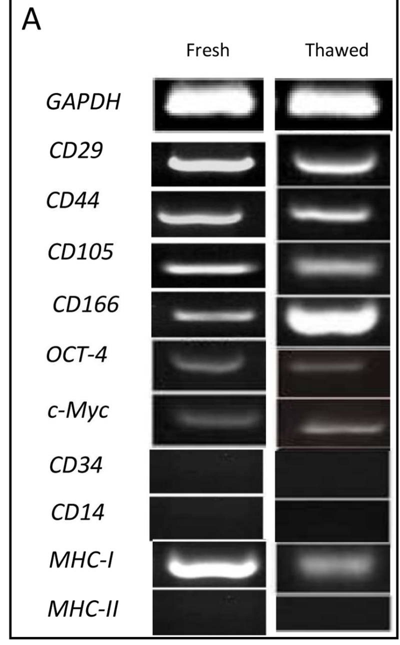

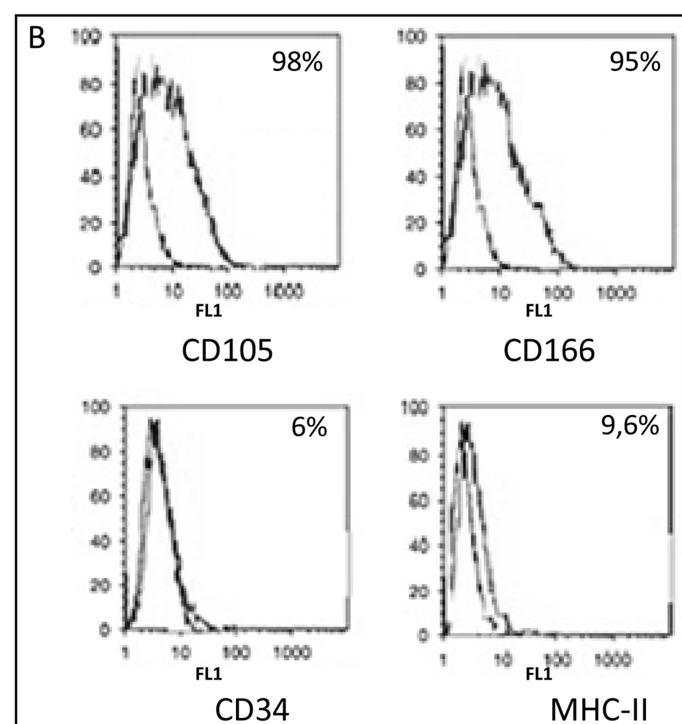

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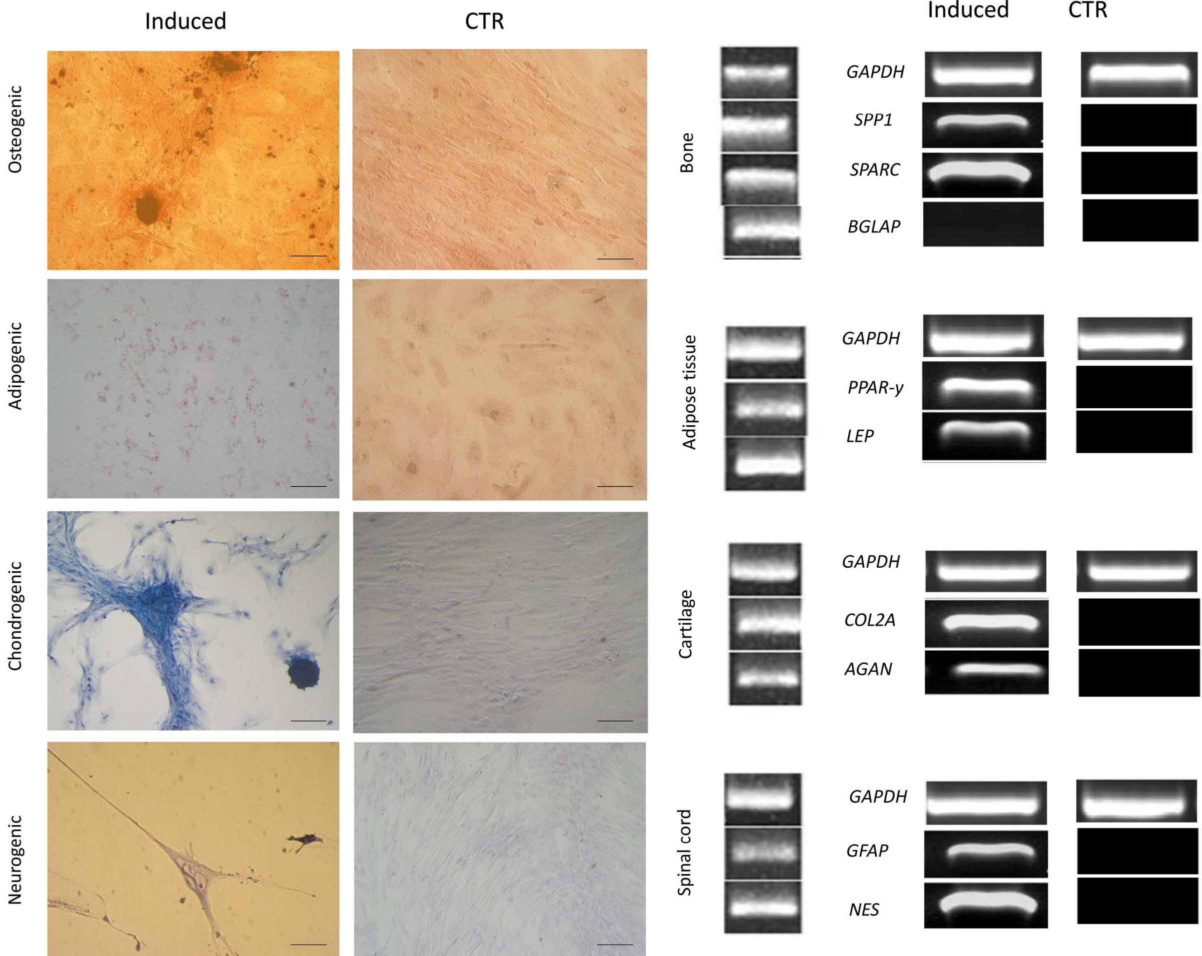
Induced
CTR
Osteogenic
Adipogenic
Chondrogenic
Neurogenic
Induced
CTR
Bone
GAPDH
SPP1
SPARC
BGLAP
Adipose tissue
GAPDH
PPAR-y
LEP
Cartilage
GAPDH
COL2A
AGAN
Spinal cord
GAPDH
GFAP
NES

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Bovine Wharton's jelly (WJ)-derived cells were isolated for the first time by enzymatic method

Molecular biology analyses revealed that these cells showed the CD antigen profile of MSCs

These cells possessed ability to differentiate in mesodermic and ectodermic lines

Their negativity to *MHC-II* reinforce the role of these cells as an allogenic source