

1 Expansion under hypoxic conditions enhances the chondrogenic
2 potential of equine bone marrow-derived mesenchymal stem cells

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13

14 **Abstract**

15 Bone marrow-derived mesenchymal stem cells (BM-MSCs) are widely used
16 in regenerative medicine in horses. Most of the molecular characterisations
17 of BM-MSCs have been made at 20% O₂, a higher oxygen level than the
18 one surrounding the cells inside the bone marrow. The present work
19 compares the lifespan and the tri-lineage potential of equine BM-MSCs
20 expanded in normoxia (20% O₂) and hypoxia (5% O₂). No significant
21 differences were found in long-term cultures for osteogenesis and
22 adipogenesis between normoxic and hypoxic expanded BM-MSCs. An up-
23 regulation of the chondrogenesis-related genes (*COL2A1*, *ACAN*, *LUM*,
24 *BGL*, and *COMP*) and an increase of the extracellular sulphated
25 glycosaminoglycan content were found in cells that were expanded under
26 hypoxia. These results suggest that the expansion of BM-MSCs in hypoxic
27 conditions enhances chondrogenesis in equine BM-MSCs

28

29 **Keywords:** Equine, Mesenchymal stem cells Bone marrow, Hypoxia
30 Chondrogenesis

31

32 Physiological oxygen concentrations surrounding cells are much lower than
33 the oxygen concentrations used in culture. Traditional incubators are
34 supplied by room air that has an oxygen content of approximately 20%
35 (referred to as 'normoxia' according to the conventional terminology). This is
36 in contrast with the oxygen tension inside the mesenchymal stem cell (MSC)
37 niches in the bone marrow (BM) which ranges between 1% and 7%,
38 (Mohyeldin et al., 2010). Hypoxia is important for the formation of cartilage
39 during fetal development. In vitro MSC chondrogenesis is controversial; in
40 some studies it is enhanced (Zscharnack et al., 2009), while in other studies
41 the opposite effect is described (D'Ippolito et al., 2006).

42 The use of MSCs as a therapeutic tool in the treatment of musculoskeletal
43 defects in horses has increased during the last few years (Frisbie and Smith,

44 2010). Most in vitro studies of equine MSCs conducted recently (Toupadakis et
45 al., 2011) have been conducted in traditional incubators to develop an
46 understanding of the MSC characteristics necessary for their use in cell therapy.
47 However, how low oxygen tension affects the expansion of equine MSCs is
48 unknown. The aim of the present study was to analyse the effect of oxygen
49 tension on the proliferation and differentiation potentials of equine MSCs
50 derived from BM (BM-MSCs), comparing cells expanded under hypoxic and
51 normoxic conditions.

52 BM aspirates were obtained from a total of five castrated male horses. All
53 procedures were carried out under Project Licence PI36/07 approved by the
54 Ethics Committee for Animal Experiments from the University of Zaragoza.
55 The care and use of animals was performed in accordance with the Spanish
56 Policy for Animal Protection RD1201/05, which meets the European Union
57 Directive 86/609 on the protection of animals used for experimental and other
58 scientific purposes.

59 Samples were collected and MSC were isolated as previously described
60 (Ranera et al., 2011a,b). Cultures were maintained at 37 °C in either normoxic
61 (20% O₂) or hypoxic conditions (5% O₂) in growth media. Growth potential of
62 equine BM-MSCs was determined for 10 passages (Appendix A;
63 Supplementary material). Equine BM-MSCs were also expanded until passage
64 2 in both oxygen conditions. The cells were then cryopreserved for further
65 analysis.

66 Osteogenic and adipogenic differentiation analyses were performed
67 (Appendix A; Supplementary material). For chondrogenic differentiation,
68 BM-MSCs were thawed and 500,000 BM-MSCs were pelleted and exposed
69 to chondrogenic induction media (Ranera et al., 2011a,b) for 21 days at 20%
70 O₂. After the induction period, the micromasses were sectioned and
71 examined by Alcian blue and safranin O staining. Immunohistochemistry for
72 S-100 protein was performed using the polyclonal rabbit antibody IR504
73 (Dako). On days 0, 7, 14 and 21 sulphated glycosaminoglycans (sGAG) and
74 gene expression of chondrogenesis-related and *HIF-1a* genes were
75 assessed. The RNA spin mini (GE Healthcare) and the Superscript kit
76 (Invitrogen) were used for total RNA isolation and reverse transcription,
77 following manufacturers' instructions.

78 Table 1 lists the genes analysed by real-time quantitative PCR (RT-qPCR). The
79 levels of gene expression were determined as previously described (Ranera et
80 al., 2011a,b). Statistics were generated using PSAW Statistics 18 software.
81 Differences in quantitative analyses between BM-MSCs expanded in normoxic
82 and hypoxic conditions were evaluated with the Student's *t* test, while ANOVA
83 was used to analyse the effect of time during differentiation. Statistical
84 significance was defined as $P < 0.05$.

85 Lifespan, osteogenic and adipogenic potentials of BM-MSCs were not affected
86 by hypoxia. No significant differences were detected between normoxia and
87 hypoxia cells in these assays (Appendix A; Supplementary material). In
88 contrast, chondrogenesis seemed to be enhanced by hypoxia. Fig. 1 shows
89 the fold change of the chondrogenic markers compared to the undifferentiated
90 cells from day 0. The production of sGAG by hypoxic BM-MSCs during
91 differentiation was greater than that of normoxic BM-MSCs (Fig. 1a). This

92 increase was significant on the last day of culture with respect to day 0.
93 Chondrogenesis was confirmed by the glu- cosaminoglycan-specific stains
94 (Alcian blue and safranin O) (Fig. 2a–d), which showed a greater
95 accumulation in the extracellular matrix in BM-MSCs expanded under hypoxic
96 conditions. Additionally, more chondrocyte differentiated cells were detected in
97 hypoxic pellets using S-100 protein immunostaining (Fig. 2e and f).

98 The fold change of expression of *COL2A1* and *ACAN* on day 21 with respect
99 to day 0 of differentiation was higher in BM-MSCs ex- panded under normoxic
100 condition (Fig. 1b and c), although signif- icant differences between the
101 expression levels on these days were only detected in hypoxic BM-MSCs. This
102 discrepancy was due to the fact that expression of these genes was three
103 times more up- regulated on day 0 in BM-MSCs expanded under low oxygen
104 con- dition (Appendix A; Supplementary material). This may be the re- sult of
105 an enhancement of *SOX9* expression in hypoxic cultures (Robins et al., 2005),
106 which is a trigger for expression of the chon- drogenic genes *COL2A1* and
107 *ACAN* (Barry et al., 2001).

108 The mRNA expression levels of the *LUM*, *BGN* and *COMP* genes
109 were higher in MSCs from cultures expanded under 5% O₂ than those that
110 were expanded under 20% O₂ over the course of differentiation. However,
111 significant differences were only detected be- tween pellets from hypoxic
112 and normoxic BM-MSCs on day 14 for *LUM* and on day 21 for *COMP*.
113 Statistically significant differences in expression of these markers were
114 observed for BM-MSCs expanded under hypoxia on day 21 relative to days
115 0 and 7 for *LUM* and rel- ative to days 0, 7 and 14 for *COMP*, suggesting
116 that the differenti- ation of equine BM-MSCs into chondrocytes is favoured
117 in a low- oxygen environment. These results are consistent with other re-
118 ports in bovine, human and murine species (Markway et al., 2010;
119 Robins et al., 2005; Zscharnack et al., 2009), which describe an
120 enhancement of chondrogenesis in MSCs in hypoxic conditions. *HIF-1a*
121 expression increased gradually during chondrogenesis in BM-MSCs
122 expanded under hypoxia with significant expression differences observed on
123 day 21 relative to days 0, 7 and 21 (Fig. 1 g). The up-regulation of *HIF-1a* in
124 normoxic BM-MSCs was not as marked as in hypoxic BM-MSCs during
125 the chondrogenic induction. In addition, the difference on the *HIF-1a*
126 expression was significantly higher in hypoxic cells than in normoxic cells
127 on day 21 (Appendix A; Supplementary material). The up-regulation of
128 chondrogenic-related genes and *HIF-1a* during chondrogenic induction in
129 hypoxic BM-MSCs might indicate that HIF-1a is an these results suggest that
130 HIF-1a could play a role in extracellular matrix formation by regulating the
131 expression of the chondro-genic-related genes. This is consistent with
132 cartilage formation models that propose HIF-1a as a key factor in cartilage
133 develop- ment and differentiation (Schipani, 2005).

134 Pre-culturing cells in an environment similar to the stem cell niche prior to
135 transplant enhances the therapeutic potential of the cells due to low oxygen
136 tension, which preserves the stem-like properties of the cells and increases cell
137 engraftment and motility (Rosova et al., 2008). In horses, MSCs have been
138 used to treat mus- culoskeletal injuries (Frisbie and Smith, 2010); however,
139 these cells had been exposed to 20% O₂ during expansion, which is not their

140 physiological oxygen condition. In the present work, to evaluate the in vitro
141 differentiation potential of BM-MSCs expanded under low oxygen tension, the
142 assays were performed under normoxia because the hypoxia might modify the
143 differentiation abilities and the effect of the oxygen during the expansion might
144 be disguised.

145 In conclusion, our results indicate that hypoxic culture conditions enhance
146 equine BM-MSC chondrogenesis, while osteogenesis, adipogenesis and
147 proliferation are not affected. Although further studies are necessary to fully
148 determine all effects of hypoxia in MSC cultures and how this may affect in
149 vivo treatments, the expansion of BM-MSCs under hypoxia appears to be a
150 more appropriate culture condition than the common normoxic expansion
151 used for clinical applications.

152

153 **Conflict of interest statement**

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

157

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166

167 **Appendix A. Supplementary material**

168 Supplementary data associated with this article can be found, in the online
169 version, at <http://dx.doi.org/10.1016/j.tvjl.2012.06.008>.

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219

220 **Tables**

221 **Table 1:** Chondrogenesis-related genes analysed by RT-qPCR. GenBank
 222 accession numbers of the sequences used for primers design.

Genes	Accession number	Primer sequence	Amplicon size (bp)
<i>COL2A1</i>	AF034691	F: CAGACGGGTGAACCTGGTAT R: TCTCCACGAGCACCTCTTTT	130
<i>ACAN</i>	AF019756	F: CTACGACGCCATCTGCTACA R: ACCGTCTGGATGGTGATGTC	96
<i>BGN</i>	AF035934	F: AAGGCCTCCAGCATCTCTATG R: GGAGATGTAGAGCTTCTGCAGC	107
<i>LUM</i>	AB292110	F: CTTGTCCATAGTGCATCTGCTTTAAG R: GAAAGTAAACGCACCTGGATTCA	100
<i>COMP</i>	AF325902	F: GGCGACGCGCAAATAGA R: GCCATTGAAGGCCGTGTAA	111
<i>GAPDH</i>	NM_001163856	F: GGCAAGTTCCATGGCACAGT R: CACAACATATTCAGCACCAGCAT	128
<i>B-2M</i>	NM_001082502	F: TCGTCCTGCTCGGGCTACT R: ATTCTCTGCTGGGTGACGTGA	102

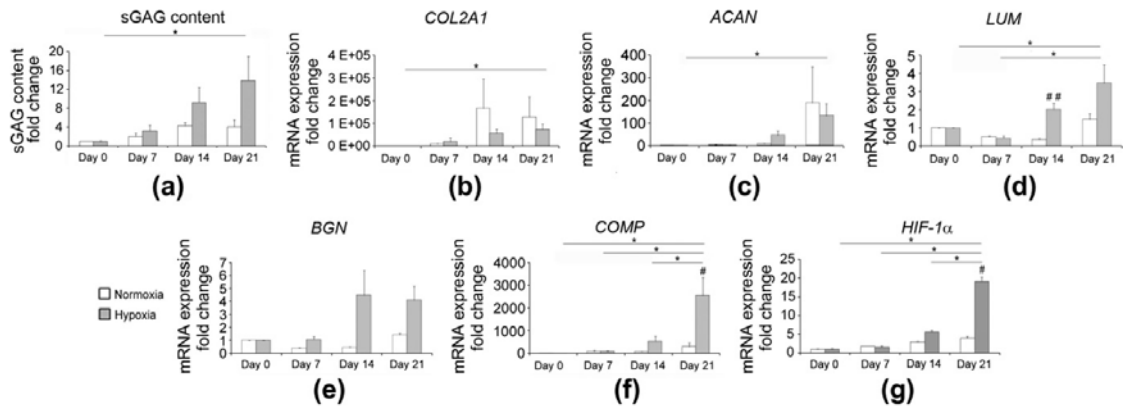
223

224 F, forward and R, reverse primers; bp, base pair.

225

226 **Figures**

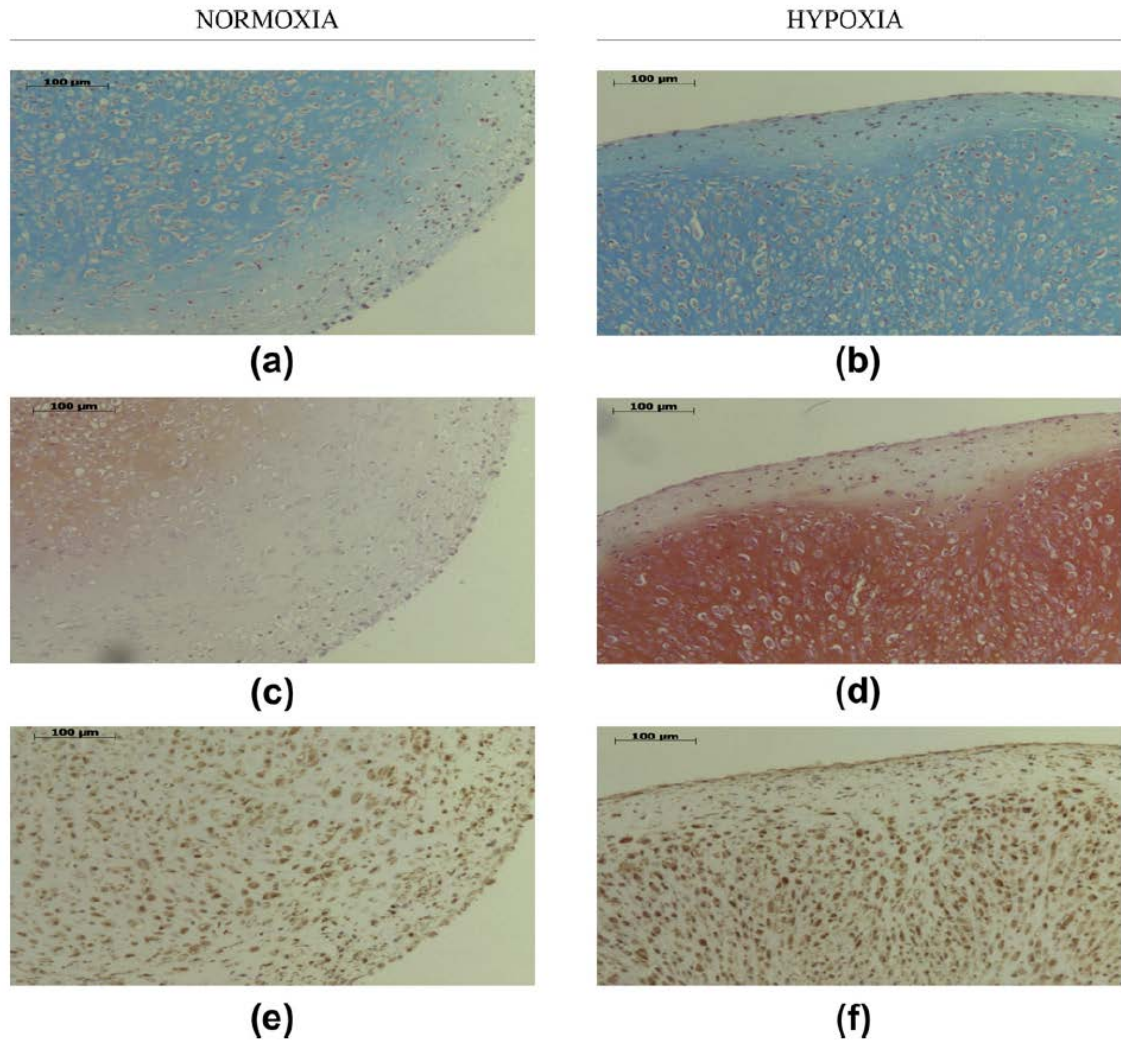
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229 **Figure 1:** Mean ± standard error of data normalised with undifferentiated cells
 230 (day 0) of bone marrow-derived mesenchymal stem cells (BM-MSCs; n = 4).
 231 The white bars represent cells expanded under 20% O₂ and the grey bars
 232 represent cells expanded under 5% O₂. (a) Sulphated glycosaminoglycans
 233 (sGAG) formed in the extracellular matrix of the pellets; (b–f) mRNA
 234 expression of chondrogenesis-related genes at days 0, 7, 14 and 21 of
 235 differentiation; (g) mRNA expression of HIF-1a at days 0, 7, 14 and 21 of
 236 differentiation. Statistically significant differences for each oxygen condition
 237 over the chondrogenesis time course, P < 0.05; statistically significant
 238 differences between normoxic and hypoxic BM-MSCs, #P < 0.05, ###P < 0.01.

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241

242 **Figure 2:** Representative staining for chondrogenic differentiation in normoxic
 243 bone marrow-derived mesenchymal stem cell (BM-MSCs; on the left) and
 244 hypoxic BM-MSCs (on the right). (a and b) Extracellular glycosaminoglycans
 245 stained by Alcian blue on day 21 of chondrogenesis; (c and d) extracellular
 246 glycosaminoglycans stained by safranin O on day 21 (magnification 5x); (e and
 247 f) S-100 immunohistochemistry on differentiated chondrocytes on day 21.

248