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Comparative evaluation of equine mesenchymal stem cells derived from amniotic fluid and umbilical cord blood

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ABSTRACT

Mesenchymal stem cells (MSCs) are promising therapeutic tools for the treatment of tendon rupture and other musculoskeletal injuries in horses. Although MSCs from bone marrow and adipose tissues are commonly used for therapeutic purpose in equines, umbilical cord blood (UCB) and amniotic fluid (AF) are potential non-invasive sources of MSCs. We collected AF and UCB from twenty mares during foaling for isolation of MSCs and evaluated them for the differences in isolation rates, proliferation capacity, expression of MSC markers and multi-lineage differentiation ability. The plastic adherent colonies were observed in 60% AF and 65% UCB samples. The mean doubling time for AF cells was significantly lower than that of UCB cells. The AF-MSCs proliferated till passage 36 whereas UCB-MSCs till passage 20 only. Both AF and UCB derived cells expressed CD29, CD44, CD73, CD90 and CD105 and were negative for haematopoietic and leukocytic markers (CD14, CD34 and CD45). The CD90 and CD73 expression was significantly higher in AF derived cells as compared to UCB-MSCs. On the other hand, CD29 expression was significantly lower in AF derived cells as compared to UCB derived cells. The UCB-MSCs differentiated poorly to adipogenic lineage compared to AF-MSCs. These results suggested that equine AF yields more MSCs with greater *in vitro* proliferation and differentiation capacities and is better non-invasive source of MSCs for regenerative therapies in equines.

Key words: Amniotic fluid, Comparison, Equine, Mesenchymal stem cell, Umbilical cord blood

Equine fetal adnexa (umbilical cord blood, Wharton's jelly, amniotic fluid, etc) are rich, safe and non-invasive potential sources of mesenchymal stem cells (MSCs). Amniotic fluid-derived MSCs (AF-MSCs) are thought to originate from the developing fetus and are intermediate stage between embryonic stem cell and lineage-restricted adult stem cells (De *et al*. 2007). Owing to their multi-lineage differentiation potency (Gulati *et al*. 2013, Iacono *et al*. 2012, Park *et al*. 2011), AF-MSCs may be a readily available source for large numbers of different cell progenitors (De *et al*. 2007). The umbilical cord blood derived MSCs (UCB-MSCs) represent the second major source of stem cells (Pappa and Anagnou 2009) and are unique as they possess an intermediate phenotype that more closely resembles embryonic stem cells (ESCs) (Reed and Johnson 2008). No information is available on the comparative evaluation of AF and UCB as source of MSCs.

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In this manuscript, we focus on the comparison of mesenchymal stem cells derived from AF and UCB. An important question to be answered is whether MSCs from different sources are comparable in their *in vitro* proliferation, expression of MSC surface markers and multi-lineage differentiation potential or whether this potential is influenced by the niche from where they were obtained. Our findings might be useful in selection of the suitable MSC type for equine regenerative cellular therapy.

MATERIALS AND METHODS

Unless otherwise specified, all the chemicals and cell culture media used for mesenchymal stem cell isolation and culture were procured commercially.

Sampling: AF and UCB were obtained from thoroughbred mares (20) maintained in an organized farm near Hisar, Haryana. AF samples (about 60 ml) were aspirated aseptically from the amniotic sac protruding from the vulva before its spontaneous rupture during the full-term foaling using a sterile 18 gauge needle attached to a 60 ml sterile syringe and collected in a 100 ml sterile container containing one ml each of EDTA and antibiotic anti-mycotic solution. UCB (130–200 ml) was collected before spontaneous breakage of umbilical cord via venipuncture using 2.5–cm, 16-gauge needle into a blood

collection bag containing citrate-phosphate dextrose-adenine (CPDA) as the anticoagulant solution, after clamping and disinfecting the umbilical cord with 70% alcohol. Samples were stored and transported at 4ºC and further processed within 4 h.

Isolation, culture and expansion of mesenchymal stem cell: Cells derived from equine AF and UCB were cultured for isolation of MSCs as previously described (Gulati *et al.* 2013, Mohanty *et al*. 2014).

Population doubling time (PDT): The proliferation capacity of AF and UCB derived cells was evaluated at passage P1 to P8 in triplicates from three different donors. In each passage, 5×10^3 cells/cm^2 were cultured in 25 cm^2 tissue culture flask. At 80% confluency, cells were trypsinized and the number of viable cells was counted by the trypan blue dye exclusion method. The population doublings (PD) were obtained according to the formula CD = ln (Nf/Ni)/ln2, PD = CT/CD, where Ni represents initial seeded cells, Nf is the final number of cells harvested, CT is the culture time (in h) and CD is the cell doubling number.

Plating efficiency and growth kinetics: Cells from both sources were seeded (300 cells/cm^2) in the growth medium in 60 mm tissue culture dishes in triplicate and incubated for 5 days. Cells were then fixed with methanol for 30 min and stained with Giemsa stain for 15 min. Colonies consisting of more than 16–20 nucleated cells, were counted and data was reported as plating efficiency (PE %), calculated as

PE % = number of colonies/number of seeded cells × 100.

AF and UCB derived cells at passage 4 were plated at a density of 1.8×10^4 into 6 well tissue culture dishes. Every 2 days, over the 10 days of culture, cells from one well of each plate were trypsinized and counted. All the experiments were performed in triplicate. At each time point cell growth curve was plotted to show an increase in cell concentration.

Reverse transcription polymerase chain reaction (RT-PCR): The expression of MSC surface marker genes (CD73, CD90 and CD105) and haematopoeitic/ leukocytic marker genes (CD14, CD34 and CD45) was assessed by RT-PCR at passage 3 using primers (Table 1) as described (Gulati *et al.* 2013, Mohanty *et al.* 2014).

Analysis of cell surface protein markers: Flow cytometry was performed in triplicates on three different MSC samples from both the sources for expression of MSC markers i.e. CD29, CD44, CD73, CD90 and haematopoietic/leukocytic marker, viz. CD34 and CD45 at passage 3 and 6. The cells were trypsinized and re-suspended 10^6 cells/ml in culture medium. The cells were pelleted and fixed with 4% paraformaldehyde at 4ºC followed by two washings with washing buffer (0.2% BSA in PBS containing 0.01% sodium azide). Cells (10^5) in triplicate tubes were incubated with 1: 50 dilution of mouse anti-human CD29-FITC,

Table 1. Details of primers used for RT-PCR analysis

Marker	Primer sequence (5'-3')	Amplicon size (bp)	Annealing temp. (ºC)	Accession number
GAPDH	F: CAAGGTCATCCATGACAACTTTG R: GTCCACCACCCTGTTGCTGTAG	496	58	NM_001163856.1
CD73	F: GGGATTGTTGGATACACTTCAAAAG R: GCTGCAACGCAGTGATTTCA	90	60	XM 001500115.2
CD90	F: TGCGAACTCCGCCTCTCT R: GCTTATGCCCTCGCACTTG	93	60	EU881920.1
CD105	F: AAGAGCTCATCTCGAGTCTG R: ATGCTCAGGGATCATTGGGG	338	56	XM_003364145.1
CD34	F: CACTAAACCCTCTACATCATTTTCTCCTA R: GGCAGATACCTTGAGTCAATTTCA	101	60	XM 001491596
CD45	F: TGATTCCCAGAAATGACCATGTA R: ACATTTTGGGCTTGTCCTGTAAC	101	60	AY114350.1
Leptin	F: GCACTGTGGACCCCTGTGC R: TGGAGGAGACTGACTGCGTG	158	62	NM_001163980.1
Adiponectin	F: GGAGACAGCTACTCCCCAAGAT R: GTCCAGTCTTACCTCTCAAACCT	187	58	NC_009146.2
Decorin	F: CTTGCACAAGTTTCCTGGGC R: CGCTTTTCGCACTTTGGTGA	481	62	NM_001081925.1
Tenomodulin	F: ACATGGAAATTGATCCCGTG R: GTCTTGTAACTCTGAAACTGC	363	58	NM_001081822.1

CD44–FITC, CD73-FITC, CD90-PE, CD45-FITC and CD34-PE antibodies for 45 min in dark at room temperature. Cells were washed 3 times and re-suspended in washing buffer. For all tubes, at least 10,000 cells were analyzed using FACS Calibur. The data were analyzed with FACSDiva software. All data were corrected for non-specific binding using isotype-matched negative controls.

In vitro multi-lineage differentiation: Differentiation of both AF-MSCs and UCB-MSCs to osteocytes, chondrocytes, adipocytes and tenocytes were induced in triplicate on 3 independent MSC samples at passage 3 and 5. The cells were re-suspended in MSC growth medium at a density of 3×10^3 cells/cm^2 in 6-well tissue culture dishes and incubated at 5% CO_2, 38.5ºC. On attaining 80% confluency, the cells in triplicate wells were incubated in respective differentiation media for 28 days with medium changes every 3rd day. The cells cultured in MSC culture medium containing 2% fetal bovine serum for 28 days served as undifferentiated control.

For osteogenic differentiation, the medium consisted of low glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 100 nM dexamethasone, 10mM β-glycerophosphate and 0.05 mM L-ascorbic acid-2-phosphate. The cells were stained with Von Kossa and Alizarin red S stain on day 14, 21 and 28.

For chondrogenic differentiation, the medium comprising high glucose DMEM supplemented with 1% ITS-Prepix, dexamethasone (1μM), ascorbic acid-2-phosphate (0.1μM), L-proline (40 μg/ml), sodium pyruvate (1 mM) and human recombinant transforming growth factor β3 (TGFβ3) at 10 ng/ml was used. The differentiated cells were stained with 1% Alcian blue (in 3% acetic acid, pH 2.5) on day 14, 21 and 28 (Table 1).

For adipogenic differentiation, the cells were cultured in adipogenic induction media for 72 h followed by culture in adipogenic maintenance media for 24 h. Adipogenic induction medium consisted of low-glucose DMEM supplemented with 10 % FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX), 0.2mM indomethacin, 1% penicillin/streptomycin and different concentration (10–20μg/ml) of recombinant human insulin, rabbit serum (10–20 %). Adipogenic maintenance media contained same ingredients as adipogenic induction media except IBMX. The differentiated cells were stained with Oil-Red-O (0.5% in isopropanol) followed by counter staining with Harris' haematoxylin for 1 min. RNA isolated from the cells was tested by RT-PCR for expression of *Leptin* and *Adiponectin* genes using gene specific primers (Table 1).

To induce tenogenic differentiation, bone morphogenic protein-12 (BMP-12) was supplemented at 50 ng/ml to MSC growth medium. The cells were stained by haematoxylin and eosin (H and E) staining for morphology study. The expression of tenocyte specific markers i.e. tenomodulin (Tnmd) and decorin (Dcn) was confirmed by gene expression in RT-PCR and protein expression by immunocytochemistry and flow cytometry. The undifferentiated MSCs served as negative control. For gene expression, RNA was isolated from differentiation induced cells as well as undifferentiated MSCs, processed for RT-PCR with tenomodulin and decorin specific primers (Table 1). For flow cytometry, polyclonal antibodies raised against peptides of human tenomodulin (N-14) and decorin (N-15) in goat were used as primary antibody and FITC conjugated donkey anti-goat IgG as secondary antibody following the protocol described in previous sections.

Statistical analysis: The SPSS 17.0 software package was used for the statistical analysis. All the data were presented as mean ± S.E. Data were analyzed by one-way ANOVA using Duncan's multiple range test (DMRT) at 0.05% level of significance.

RESULTS AND DISCUSSION

Isolation and proliferation of MSCs: The plastic adherent fibroblastoid spindle-shaped cells growing in a monolayer could be isolated from 12 of 20 AF samples and 13 out of 20 UCB samples, with isolation rates of 60% and 65%, respectively, which was comparable to an earlier report (Iacono *et al*. 2012). In the present study, both AF and UCB were easily collected at the delivery, without any invasiveness, and high numbers of viable nucleated cells from these sources were isolated, except for some AF samples due to bacterial contaminations. Non-sterile partum canal and prevailing environmental conditions are limiting factors for aseptic collection of amniotic fluid. Similar observations have been reported by other authors (Iacono *et al*. 2012).

On initial culture, about 80% confluency was reached after 17 days post-seeding in AF samples whereas, it was around 30 days in UCB samples. During initial culture, AF-MSCs were more homogenous compared to UCB derived MSCs which was confirmed by alkaline phosphatase staining (data not shown). The different morphologies in initial culture might reflect mixture of true mesenchymal stem cells with unrestricted somatic stem cells (Koch *et al.* 2007, Corradetti *et al*. 2013).

Population doubling time for initial 8 passages in cells from both sources was compared (Table 2). The AF derived cells had an average cell doubling time of 43.33±0.91 h which was significantly ($P\leq 0.05$) lower than UCB cells (46.40 ± 2.86 h) (Table 2), as reported earlier (Iacono *et al*. 2012). During initial 8 passages, average plating efficiency for AF-MSCs was comparatively lower than that for UCB-MSCs (Table 2). The calibrated growth curve at passage 4 was 'S' shaped with a short lag phase in both the MSCs. The average plating efficiency during initial 8 passages showed that UCB-MSCs had more clonogenic property than AF-MSCs (Lovati *et al*. 2011). AF-MSCs were able to proliferate till passage 36, whereas UCB-MSCs divided till passage 20. These behaviors could reflect the more primitive nature of AF-MSCs when compared to cells isolated from UCB-MSCs.

MSC characterization: In RT-PCR, both AF and UCB derived cells displayed gene expression for the

Table 2. Comparison of population doubling time and plating efficiency between AF-MSCs and UCB-MSCs at different passages

Passage number	Population doubling time (h)		Plating efficiency (%)	
	AF-MSC	UCB-MSC	AF-MSC	UCB-MSC
1	47.51±0.23	30.48±1.43	3.11±0.01	2.53±0.33
2	45.80±0.28	47.52±0.48	2.58±0.10	2.17±0.02
3	44.32±0.11	45.16±0.01	2.27±0.13	1.61±0.01
4	43.30±0.23	46.02±0.02	2.18±0.13	2.61±0.48
5	39.57±0.05	45.225±0.54	2.26±0.03	3.50±0.06
6	40.63±0.44	45.91±0.11	2.03±0.04	2.96±0.04
7	42.30±0.28	53.59±0.59	1.98±0.06	2.38±0.08
8	43.27±0.17	58.53±0.44	1.92±0.02	2.66±0.26
Mean	43.33±0.91	46.40±2.86	2.29±0.14	2.55±0.22

Data are presented as mean ± SE.

Table 3. Summary of MSC surface markers expression by amniotic fluid and umbilical cord blood derived cells using flow cytometry

Marker	% Positive (mean±SE)	
	AF	UCB
CD90	93.70 ± 1.89	46.75 ± 3.95
CD73	96.96 ± 0.44	73.23 ± 1.29
CD29	68.83 ± 1.27	96.36 ± 1.28
CD44	93.66 ± 1.80	93.40 ± 0.70
CD34	2.75 ± 1.55	2.40 ± 0.20
CD45	5.20 ± 2.40	0.10 ± 0.0

mesenchymal surface markers CD73, CD90 and CD105 at P3, while haematopoietic and leukocytic markers CD14, CD34 and CD45 were not registered at P3 for either population (Fig.1). The results are in accordance with earlier reports for adipose tissue and bone marrow derived MSCs (Ranera *et al*. 2011). However, variable results for CD34

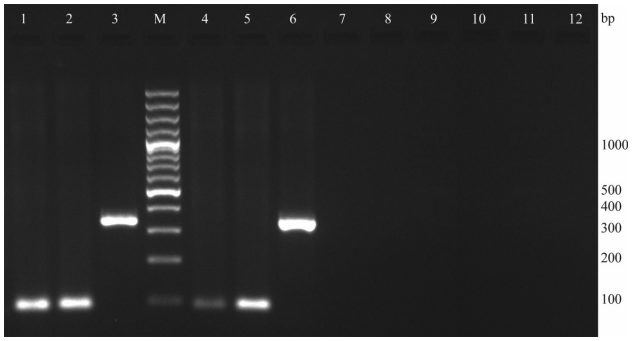

Fig. 1. Gene expression analysis of mesenchymal and haematopoietic cell surface markers in equine AF and UCB derived cells. Both AF- and UCB-derived cells showed the expression of mesenchymal markers CD73 (lane 1 and 4), CD90 (lane 2 and 5), CD105 (lane 3and 6), but did not express haematopoietic/ leukocytic markers CD14 (lane 7 and 10), CD34 (lane 8 and 11) and CD45 (lanes 9 and 12), respectively. M, 100 bp ladder; bp, base pair.

expression by equine bone marrow and amniotic membrane derived MSCs have been reported previously (Lange-Consiglio *et al*. 2012, Ranera *et al*. 2011).

Flow cytometry analysis of AF and UCB derived horse MSCs with cross-reactive antibodies against cell surface mesenchymal and haematopoietic antigens is summarized in Table 3. The CD90 and CD73 expression was significantly higher in AF derived cells than that in UCB-MSCs. On the other hand, CD29 expression was significantly lower in AF derived cells than that in UCB derived cells. Reactivity against the haematopoietic antigens CD45 and CD34 was ≤ 5% in both AF and UCB derived cells. In the present study, both AF-MSCs and UCB-MSCs positively expressed cell surface mesenchymal markers, similar to previous reports (De *et al*. 2012, Iacono *et al*. 2012), however, percentage of cells showing expression of these markers were variable, suggesting that stem cell

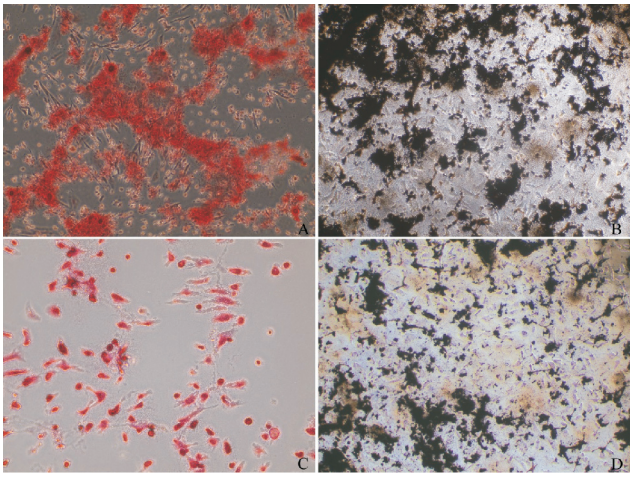

Fig. 2. Alizarin Red S (A, C) and Von Kossa (B, D) staining after osteogenic differentiation of AF-MSCs and UCB-MSCs.

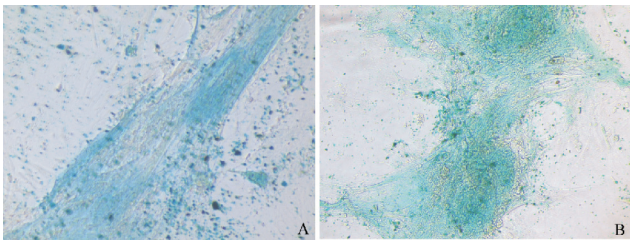

Fig.3. Alcian Blue staining after chondrogenic differentiation of AF-MSCs (A) and UCB-MSCs (B) showing marked deposition of glycosaminoglycans in the matrix.

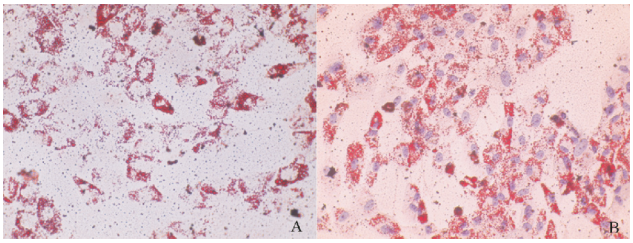

Fig.4. Oil Red O-stain after induction of adipogenic differentiation of AF-MSCs (A) and UCB-MSCs (B) showing cytoplasmic neutral triglycerides droplets.

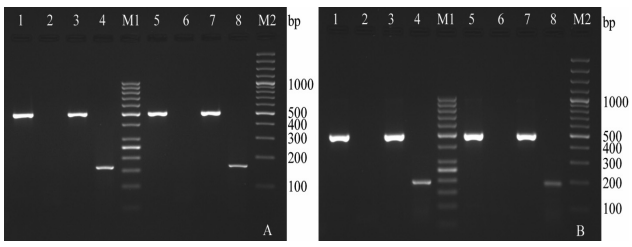

Fig. 5. Gene expression analysis of AF and UCB-MSCs after adipogenic differentiation. Leptin (A) and Adiponectin (B) genes were expressed by AF-MSCs (lane 4) and UCB-MSCs (lane 8), but not by undifferentiated control cells (lanes 2, 6), respectively. GAPDH gene was expressed by both undifferentiated control cells (lanes 1, 5) and by AF- and UCB-MSCS cells induced to osteogenic differentiation (lanes 3, 7), respectively. M1, 50 bp DNA ladder; M2, 100 bp DNA ladder; bp, base pair.

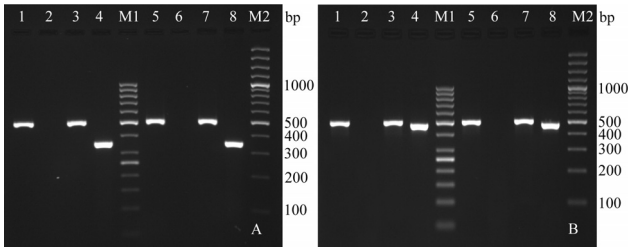

Fig. 6. Gene expression analysis of AF and UCB-MSCs after tenogenic differentiation. Tenomodulin (A) and Decorin (B) genes were expressed by AF-MSCs (lane 4) and UCB-MSCs (lane 8) induced to tenoogenic differentiation, but not by undifferentiated control cells (lanes 2, 6), respectively. GAPDH gene was expressed by both undifferentiated control cells (lanes 1, 5) and by AF- and UCB-MSCS cells induced to osteogenic differentiation (lanes 3, 7), respectively. M1, 50 bp DNA ladder; M2, 100 bp DNA ladder; bp, base pair.

property is affected by the niche from where these cells were obtained.

Multi-lineage differentiation: In the present study, we demonstrated the tri-lineage differentiation capability of equine AF-MSCs and UCB- based on the principles established by the International Society for Cytotherapy.

On induction to osteogenic differentiation, AF-MSCs were detected positive by Alizarin Red S and Von Kossa staining after 14 days of osteogenic induction, whereas UCB-MSCs were detected for positive osteogenesis as early as on day 21 post-induction (Fig. 2). UCB-MSCs were also found to change their morphology from regular fibroblastoid to relatively smaller cytoplasmic extensions and some were rounded too. In AF, cell clusters were found which represented centers of production of calcium apatite and thus centers of osteogenic differentiation (Fig. 2). Undifferentiated control cells did not take any staining.

UCB-MSCs cultured in chondrogenic differentiation media showed more chondrogenic potential than AF-MSCs by marked deposition of glycosaminoglycans (GAG) in the matrix by day 21, which was observable after Alcian Blue staining (Fig. 3). The cells maintained in regular control medium showed no detectable deposition of GAGs. The chondrogenic differentiation in UCB-MSCs was found to be more prominent previously (Burk *et al*. 2013).

The induction of adipogenic differentiation resulted in differentiation of both these MSCs to adipocytes by day 10 compared with non-induced control cells, as demonstrated by Oil Red O staining (Fig. 4). AF-MSCs showed more adipogenic differentiation potential than UCB-MSCs as observed by larger intra-cytoplasmic Oil Red O positive lipid droplets. During adipogenic differentiation, a distinct ring of dark coarse granules around the cell periphery was observed initially by day 3, which developed into fat globules by day 10 with accumulation of intracytoplasmic vacuoles of Oil red O stained neutral triglycerides droplets. The adipogenic induction was further confirmed by the presence of mRNA for leptin and adiponectin by RT-PCR after 10 days of induction (Fig. 5). Koch *et al*. (2007) reported that UCB-MSCs showed poor adipogenic differentiation compared to AF-MSCs whereas AF-MSCs showed more adipogenic differentiation potential (Lovati *et al*. 2011). Further studies are necessary to verify if in equine the conditions for AF and UCB-MSCs promote one type of differentiation lineage rather than other, as suggested (Park *et al*. 2011).

AF-MSCs and UCB-MSCs were induced to tenocytes on day 14 when cultured in the growth medium supplemented with 50 ng/ml of BMP-12. The BMP-12 treated cells appeared more slender, elongated, spindle shaped with thinner and longer cytoplasmic processes as compared to control untreated cells. RT-PCR analysis showed the expression of tenomodulin and decorin gene in differentiated tenocytes with respect to the control groups (Fig. 6). Further, flow cytometry data showed that decorin expression in AF-MSCs derived tenocytes is statistically ($P \leq 0.05$) lower than UCB cells. About 96.7±1.90% of AF-MSCs and 95.65±2.15% of UCB-MSCs expressed tenomodulin on BMP-12 treatment in comparison to 11.30±0.10 % cells in untreated control. Since the trans-membrane protein tenomodulin is expressed by mature tenocytes and has been implicated in regulating their proliferation and matrix organization (Docheva *et al*. 2005), our data suggest that AF derived tenocytes are more mature than UCB-MSCs.

To summarize, our results suggest that equine AF contains higher number of viable MSCs with greater *in vitro* proliferation and differentiation capacities, and is therefore a convenient cell source for autologous or allogeneic regenerative therapies. However, in specific clinical settings, it may be beneficial to take advantage of UCB as source of MSCs for specific differentiation capacities like differentiation to chondrocytes. Further *in vivo* studies will be required to substantiate our *in vitro* findings.

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