Indian Journal of Animal Sciences **91** (2): 114–120, February 2021/Article
https://doi.org/10.56093/ijans.v91i2.113818

Expression of reprogramming factors in mesenchymal stem cells isolated from equine umbilical cord Wharton's jelly and amniotic fluid

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Received: 10 February 2021; Accepted: 12 July 2021

ABSTRACT

Stem cells represent the most promising population for regenerative cell therapy and have gained much attention during the recent past. Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into trilineages. Like haematopoietic cells, mesenchymal cells have been shown to proliferate and form fibroblast-like colonies *in vitro*. Despite major progress in our general knowledge related to the application of adult stem cells, finding alternative sources for bone marrow MSCs has remained a challenge. A wide diversity of isolation procedures for mesenchymal stromal cells from various tissues of the placenta, umbilical cord and Wharton's jelly have been described for humans and other species. In this study, we isolated established umbilical cord Wharton's jelly as a primary source for isolation of mesenchymal stem cells since it is a rich source of stem cells and no ethical concerns are involved. Equine umbilical cord Wharton's jelly segments were collected during foaling time and digested enzymatically and cultured *in-vitro* in culture medium. In addition to the study of their morphology and colony forming units, the expression of reprogramming factors by the isolated MSCs were also studied. The isolated MSCs were observed to be plastic adherent, clonogenic and their morphology were polygonal, star shaped and fibroblast like. They revealed a strong expression of pluripotent stemness markers OCT–4, SOX–2, Nanog and KLF–4. From the current study, it can be concluded that Wharton's jelly is a rich source of stem cells with stemness properties expressing the reprogramming factors and mesenchymal like morphology and could be used as an alternate for the bone marrow derived mesenchymal stem cells for cell based regenerative therapies.

Keywords: Equine, Mesenchymal stem cells, Reprogramming factors, Umbilical cord, Wharton's jelly

The clinical use of cellular therapy and tissue engineering in veterinary medicine is developing at rapid pace (Gulati *et al.* 2015). Stem cell therapy has shown promising results in tendinitis and osteoarthritis in equine medicine (Rathore *et al.* 2018). Among adult stem cells, mesenchymal stem cells (MSCs) are reported to be able to self-renew and differentiate into cells of connective tissue lineages, including bone, fat, cartilage and muscle (Barry and Murphy 2004, Lee *et al*. 2007). MSC are non-hematopoietic, multipotent progenitor cells that are easily isolated from various adult tissues. Rich source for MSCs include bone marrow, as well as solid tissues such as adipose tissue. Previously, horse MSCs have been isolated from bone marrow (Koerner *et al.* 2006, Vidal *et al.* 2007, Arnhold *et al.* 2007, Kisiday *et al.* 2008), adipose tissue (Kisiday *et al.* 2008, Vidal *et al*. 2012), peripheral blood (Koerner *et al.* 2006) and umbilical cord blood (Koch *et al.* 2007, Reed and Johnson 2008). Although recovery of MSCs from bone marrow is a common option, there are increased concerns over the invasive aspiration procedure of bone marrow and the potential complications involved for the donor horses (Toupadakis *et al.* 2010, Kasashima *et al.* 2011). Furthermore, there are cell culture-specific limitations associated with MSCs derived from adipose tissue and bone marrow, such as limited recovery of MSCs, complications of contamination and early cell senescence associated with donor age (Cremonesi *et al.* 2008, Lovati *et al.* 2011). In comparison to bone marrow, various other tissues, such as amniotic membrane, placental membranes or umbilical cord matrix proved to yield higher numbers of MSCs that are highly proliferative and that also possess multilineage differentiation potential (D'Ippolito *et al.* 1999). 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. 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 multilineage differentiation potency (Park *et al*. 2011, Iacono *et al*. 2012, Gulati *et al*. 2013), AF-MSCs may be a readily available

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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). Till now very little work has been done and meagre information is available on expression of pluripotency markers in AF and UCB derived stem cells and their comparison in *in vitro* culture patterns. Therefore, the present study was conducted with an objective to isolate, culture and characterise the MSCs from both the sources and compare their growth patterns, colony forming units and expression of reprogramming patterns in the culture.

MATERIALS AND METHODS

The present work has been carried out at Equine Production Campus (EPC), ICAR-NRC on Equines (ICAR-NRCE), Bikaner, Rajasthan. The work was conducted after obtaining the approval of the Institutional Animal Ethics Committee, ICAR-NRCE, Hisar. All the chemicals were purchased from Sigma-Aldrich (St. Louis, Mo., USA) and the plasticware from Eppendorf (Roskilde, Denmark) unless and until mentioned otherwise.

Collection of samples: All samples (Umbilical cord Wharton's Jelly (UCB-WJ) and Amniotic fluid (AF) samples; n=5 each) were obtained during foaling from Marwari breed mares present at EPC, ICAR-NRCE. To prevent the contamination and damage to the tissues, all samples were collected using sterilized syringes, surgical gloves and equipment (Fig. 1). Amniotic fluid samples (~ 40 ml) were aspirated aseptically from the amniotic sac protruding from the vulva before its spontaneous rupture at the time of foaling using a sterile 18 G needle attached to a 50 ml sterile syringe and collected in a 50 ml sterile plastic centrifuge tube containing 1 ml each of EDTA and a mixture of antibiotic (Penicillin and Streptomycin) anti-fungal (Amphotericin B) solution (Fig. 1B). The umbilical cord Wharton jelly's samples were washed twice in 5% betadine solution and then with 70% alcohol and kept at 4°C in Dulbecco's Phosphate Buffer Solution (DPBS) containing antifungal and antibiotic medium and were processed within 4 h after their collection for isolation of MSCs.

MSCs isolation and culture: For recovery, isolation and culture of the MSCs from the UCB-WJ tissue pieces and AF the following methods were adopted.

Isolation and culture of MSCs from UCB-WJ tissues: Under laminar hood, the UCB-WJ samples were mechanically minced into small pieces and subjected to enzymatic digestion as per the protocols described previously by Rathore *et al.* (2018). The UCB-WJ tissue pieces were diluted 1 : 1 with DPBS containing 100 IU/ml penicillin and 100 mg/ml streptomycin and to which we added 1 ml/1 g sample of a digestion solution (2% Trypsin and 0.1% (w/v) collagenase type I) and the samples were incubated 37°C for 1–2 h. The tissue and digestion solution were mixed thoroughly at every 15 min. After incubation, the enzymes were inactivated by adding FBS and sieved to avoid the undigested tissue particles. The obtained solution was centrifuged at 650× g for 4 min at 4°C in a refrigerated centrifuge to obtain cell pellet. The supernatant was discarded and the cell pellet was suspended in 1 ml of culture media containing DMEM with low glucose and L-Glutamine (DMEM), 20% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), sodium pyruvate and 1% antibiotic solution. Cell culture medium was additionally supplemented with 0.5 µg/ml amphotericin B until first passage to prevent any fungal contamination of the cultures. Cells were incubated at 37.5°C in a 5% CO_2 atmosphere and the culture medium was changed twice a week. After 44–48 h of culture, the medium was removed and the cells were replenished with fresh medium. The confluent cultures were harvested using 0.25% trypsin - EDTA and passaged at 1 : 2 ratios into fresh culture flasks. Sub-culture was repeated till passage 5 until sufficient cells were provided for the next stage of experiments. The cell number was counted using haemocytometer.

Isolation and culture of MSCs from AF samples: Each AF sample was diluted 1: 1 with DPBS containing 100 IU/ml penicillin and 100 mg/ml streptomycin. The obtained solution was centrifuged for 20 min at 650× g. Supernatant was carefully removed and the pellet was resuspended in 5 ml of DPBS. Cells were isolated by loading the sample on 5 ml of histopaque solution in a 15-ml polypropylene tube and centrifugation at 650× g for 10 min at 4°C. The supernatant was discarded and interphase was

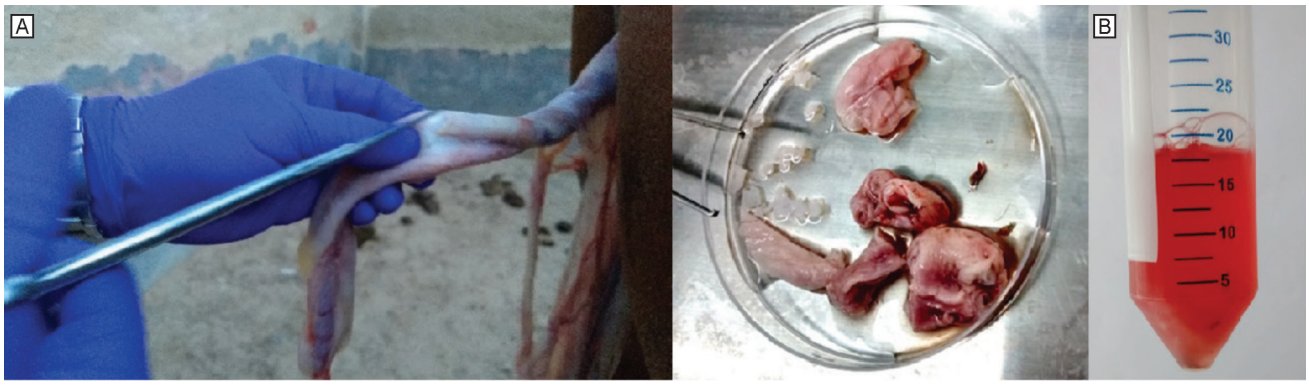

Fig. 1. Isolation of UCB-WJ samples (A) and collection of amniotic fluid (B) in culture media at the time of foaling.

collected, washed twice with DPBS and centrifuged again at 650× g for 10 min at 4°C. The cells were suspended in 1 ml of MSC growth medium as followed for the UCB-WJ MSCs.

Live cells were counted by trypan blue dye (0.4%) exclusion using a haemocytometer, seeded at 1×10^6 cells/ml in 25 cm^2 tissue culture flasks and incubated at 37.5°C in humidified atmosphere containing 5% CO_2. Initially, the medium was replaced after 48 h and thereafter every 3rd day. Cell growth and morphology was observed under an inverted microscope. The cells were detached at 80% confluency with 0.05% (w/v) trypsin, counted with a haemocytometer and re-seeded as passage 1 (P1) at 1×10^6 cells/ml in 25 cm^2 tissue culture flasks.

Population doubling time: Population doubling time was calculated by seeding cells (5×10^3 cells/cm^2) in 25 cm^2 tissue culture flasks and incubation till 80% confluency. The cells were trypsinised and the number of viable cells were counted and population doubling time (in h) was calculated as per Rathore *et al.* (2018). MSCs were seeded (300 cells/cm^2) in the growth medium in 60 mm tissue culture dishes and incubated for 5 days at 5% CO_2 and 37.5°C. The cells were then fixed with methanol and colonies consisting of more than 20 cells were counted; data were reported as plating efficiency, which was calculated as number of colonies/number of seeded cells ×100.

Cryopreservability of MSCs and their recovery: The MSCs obtained from both the samples (UCB-WJ and AF) were subjected for cryopreservation after Passage 5. The cryopreservation of cells was performed as per protocol described by Rathore *et al.* (2018). Briefly, the confluent cultures were trypsinised as above and washed thrice by centrifugation using cell culture medium. The cell pellet obtained after removal of the supernatant was mixed with 1 ml of cryopreservation media (DMSO and Culture media) and were initially stored on –80°C and shifted to LN_2 storage cryocans on next day. After 7 days of cryopreservation, the cryovials were taken out and warmed by placing them in a water bath (37°C) for ~15 sec. The cell suspension was washed to remove residual cryopreservation medium. The cells at a density of 1×10^6 cells/ml were re-cultured in 25 cm^2 cell culture flask. A fraction of cell volume (~50–100 µl) was used to evaluate the cell survival rate using trypan blue (0.4%) staining and counting live (unstained)/dead (blue stained) cells.

Viability assay: The cells isolated from the both techniques were used for determining the cell viability using trypan blue staining as described previously (Weingartl *et al.* 2002). The number of dead cells was determined from a field of 500–1000 cells.

Colony forming unit (CFU) assay: The CFU assays were done as per the methods described by Rathore *et al*. (2018). Briefly, CFU assays were performed at passage 0, 1 and 2 on freshly isolated cells at different densities (100, 250, 500 and 1000 cells/cm^2). Cells were plated in six well plates and cultured in 5% CO_2 and 90% humidity at 37.5°C for 2 weeks in DMEM medium. The colonies of MSCs were washed with DPBS and fixed with 4% formalin. These colonies were later stained with 1% methylene blue in 10 mM borate buffer, and washed twice at room temperature. Colonies formed by 20–25 nucleated cells were counted under 10× through microscope (Nikon 80i, Japan).

Growth curve: Cells of the sixth passage were seeded in six-well plates at a density of approximately 2×10^5 cells/well and cultured continuously for 9 days counted every day. The average cell counts at each time point were then plotted against time and the population doubling time was determined based on this curve (Kim *et al.* 2005).

Characterisation of the MSCs: The MSCs isolated from the both the protocols were characterized for the alkaline phosphatase (AP) staining (Talluri *et al*. 2015) and for the expression of pluripotency genes expression through RT-PCR.

Alkaline phosphatase staining: To characterise the undifferentiated state of AF-derived cells, we analysed the level of alkaline phosphatase (AP) expression. Cells at P1, P5 and P10 were subjected to AP staining using an AP staining kit (Sigma) by staining with naphthol and fast blue B alkaline solution followed by counter-staining with neutral red. Adult equine ear pinna fibroblast cells were taken as negative control.

Expression of pluripotent marker genes by reverse transcriptase polymerase chain reaction (qPCR): Expression of pluripotency genes was analysed by qPCR as per the standard protocol. The sequence of the genes used for the qPCR have been listed in the Table 1. Briefly, the total RNA was extracted from the UBWJ-MSCs and AF-MSCs at passage 5 using QiagenRNeasy kit (Qiagen, USA). The RNA was treated with DNase and then 0.2 to 1µg of total RNA was reverse transcribed to cDNA by using high capacity cDNA synthesis kit (Applied Biosystems, USA). The PCR reactions was performed in a 25 µl reaction solution using Ampliqon red dye master mix (Ampliqon,

Table 1. List of pluripotency genes and their sequences used for the current study

Marker	Forward and reverse	Sequence (5'-->3')	Amplicon size	Annealing temp (°C)
eqOCT4	Forward	GGGACCTCCTAGTGGGTCA	318	58.0
	Reverse	TGGCAAATTGCTCGAGGTCT		
eqSOX2	Forward	CACCCACAGCAAATGACAGC	252	55.0
	Reverse	TTTCTGCAAAGCTCCTACCG		
eqNANOG	Forward	TCCTCAATGACAGATTTCAGAGA	323	54.5
	Reverse	GAGCACCAGGTCTGACTGTT		

Denmark) (150 mM TrisHCl, 4 mM $MgCl_2$, 0.4 mM dNTP and 0.05 U Taq Polymerase), 20 pmol primers and 3 µl cDNA. The PCR conditions were as follows: initial denaturation for 5 min at 94°C followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec, and final extension for 10 min at 72°C. The primer sequences used for various stem cell marker genes, annealing temperatures and product size are presented in Table 1. Amplified DNA fragments were separated on 2% agarose gel containing 0.1 µg/µl ethidium bromide and visualized under UV light.

Statistical analysis: Statistical analysis was performed using Statistical Package for Social Science (SPSS® Version 20.0 for Windows®, SPSS Inc., Chicago, USA). The means were compared using analysis of variance, Duncan's multiple range test and presented as mean±standard error (SE) at the significance level of $P<0.01$ or $P<0.05$. Cell doubling time was analyzed by one-way analysis of ANOVA test. Data are expressed as $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Morphological assessment of MSCs: The MSCs recovered from the UCB-WJ and AF were observed to be plastic adherent, elongated, spindle shaped and fibroblastic as described for horses (De Schauwer *et al.* 2011, Gulati *et al.* 2012, Rathore *et al*. 2018) and human (Bieback *et al.* 2004). These MSCs at the beginning of culture (P_0) adhered to the plastic and presented endotheloid and fibroblastoid morphologies (Fig 2a). The different morphologies might be due to the presences of cells of different lineages in the placenta comprising of different tissues (Koch *et al.* 2007). During subsequent culture, there was predominance of spindle-shaped fibroblastic morphology. After reaching confluence, some of these cells grew upward and detached from the substratum surface. These characteristics were also reported in equine umbilical matrix MSCs (Lovati *et al.* 2011). The MSCs isolated from AF have multiplied vigorously after initial 2 to 4 passages and they were plastic adherent and displayed various morphological structures like elongated, spindle shaped fibroblastoid cell types (Fig. 1b). The MSCs (UCB-WJ and AF) were cultured and propagated *in-vitro* up to 35 passages which demonstrated their multiplication and clonal properties. These MSC were cryopreserved and even after thawing and re-culturing the morphology and survivability of the cells did not vary significantly. The cells displayed normal karyogram pattern before and after thawing and even at various stages of *in-vitro* culture which is indicative of further use of the cells in downstream applications.

Population doubling time: 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 ($P<0.05$) lower than UCB-WJ 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-WJ 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.

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-WJ derived MSCs which was confirmed by alkaline phosphatase staining (Fig. 2). 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).

MSCs growth patterns and their analysis: The growth curve of cells isolated (from both the protocols) from equine

Table 2. Population doubling time in MSCs isolated from UCB-WJ and AF

Passage number	Population doubling time (h)	
	UCB-WJ MSCs	AF-MSCs
1	56.47±1.18	45.81±2.06
2	59.55±1.93	50.64±1.09
3	52.183±3.19	48.29±3.01
4	50.91±5.82	55.83±0.91
5	60.38±5.51	54.07±0.22
6	59.01±4.01	50.16±1.50
7	55.93±3.05	49.29±0.36
8	57.60±5.21	55.21±1.03
9	50.91±1.05	50.88±1.93
10	63.48±2.93	51.54±1.14
Mean±SE	58.93±3.02	51.18±1.20

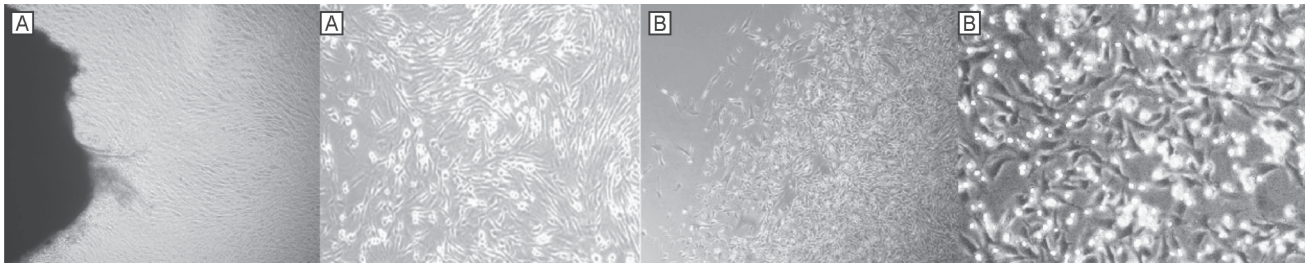

Fig. 2. Morphology of (A) UCB-WJ MSCs and (B) AF-MSCs at the time seeding (A&B) of culture and at (A^1 and B^1) P5.

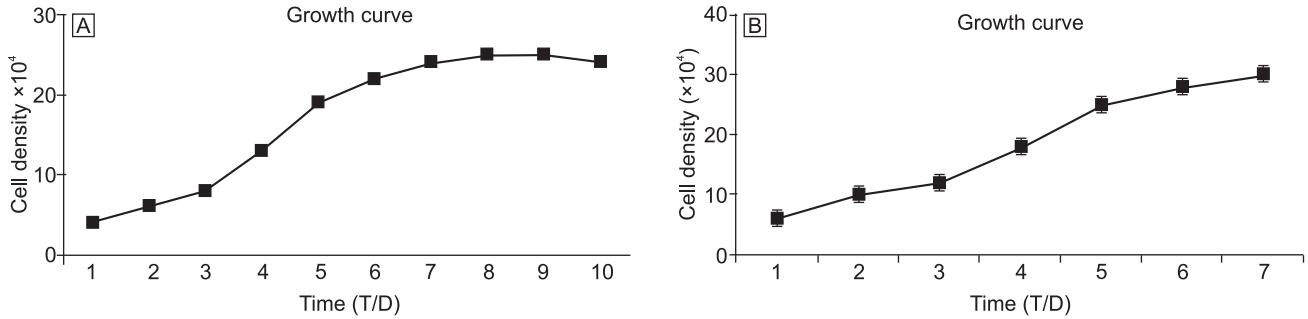

Fig. 3. Growth patterns of (A) UCB-WJ MSCs and (B) AF-MSCs.

umbilical cord Wharton jelly tissue, and of amniotic fluid had shown a classic "S" shape involving a lag, log and plateau phases (Fig. 3). The population doubling time observed was approximately 38.6 h and 40.2 h for the AF-MSCs and UCB-WJ MS cells. Mean population doubling time of these cells for initial 10 passages in the present study was lower than that reported by Iacono *et al.* (2012). This population doubling time was also lower than that of MSCs derived from adipose tissue (Vidal *et al.* 2008), wharton's jelly (Iacono *et al.* 2012, Gulati *et al.* 2016) and bone marrow (Vidal *et al.* 2008). In human, a lower doubling time and a greater proliferative activity are common characteristic of cells isolated from umbilical cord matrix and cord blood compared to those of bone marrow derived cells (Karahuseyinoglu *et al.* 2007). This behavior could reflect the more primitive nature of cells isolated from fetal adnexa compared to those obtained from bone marrow (Weiss and Troyer 2006, Gulati *et al.* 2016). There was a lag latency phase of about 24–26 h after initial seeding, with respect to the adaptation to the culture conditions and recovery of the cells from digestive enzyme damage effect; after that the cells proliferated rapidly and entered to log phase. As the density of the cells began to increase, proliferation and growth of the cell population was reduced by contact inhibition and due to space limitation, cells changed their morphology and the cells began to enter the plateau phase after the 8th day.

The mean plating efficiency of MSCs isolated from both the sources in this study was observed to be 4.57±1.57% during the initial 10 passages indicating their high clonogenicity, in comparison to those reported for MSCs isolated from amniotic fluid, umbilical cord matrix and bone marrow (Lovati *et al.* 2011, Gulati *et al.* 2016). This is further substantiated by the observation that these cells had short lag phase implying their rapid recovery from the damage occurring detachment by enzymatic treatment further these cells could be serially passaged upto 20 passages, demonstrating their better self-renewal potential as reported previously (Schuh *et al.* 2009).

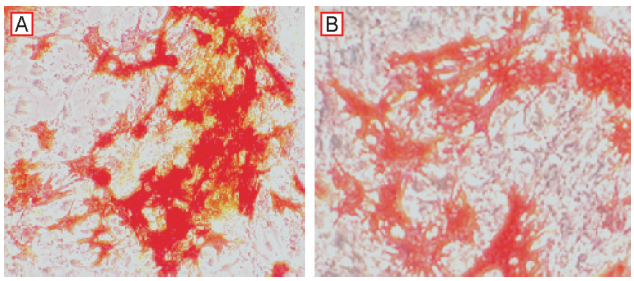

Fig. 4. Alkaline phosphate staining of (A) UCB-WJ MSCs and (B) AF-MSCs.

Characterisation of MSCs

Alkaline phosphatase staining: All the derived MSCs at different passages were subjected to alkaline phosphatase (AP) staining (Fig. 4). Cells at initial passages (P0 and P1) were heterogeneous but with subsequent sub-culturing (from second passage onwards), the cells showed homogenous AP-positive population of cells. AP positive staining demonstrated the high phosphatase activity, a unique feature of undifferentiated stem cells as reported previously (Reed and Johnson 2008, Schuh *et al.* 2009, Gulati *et al.* 2016). Although intensity of AP-staining in the present study was not compared; it has been previously reported that bone marrow derived equine MSCs took more intense AP-staining than those from adipose tissue and umbilical cord (Vidal *et al.* 2007, Guest *et al.* 2008).

Expression of reprogramming factors: A co-ordinated network of transcription factors is required for maintenance of pluripotency. Among these, *Oct-4, Nanog* and *Sox-2* interact together and central to the transcription regulatory hierarchy that specifies pluripotency (Chambers *et al.* 2003).

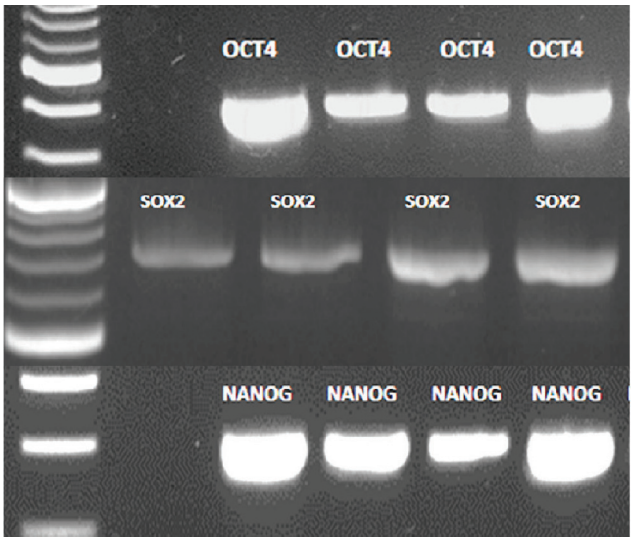

Fig. 5. Pluripotency gene expression pattern in UCB-WJ MSCs and AF-MSCs at P5 and P10.

UCB-derived MSCs in the present study showed expression of *Oct-4, Nanog* and *Sox-2* genes by qPCR (Fig. 5). But *Nanog* and *Sox-2* expression were not observed in UCB-MSCs in previous study (Reed and Johnson 2008). The expression of these three pluripotency transcription factors has been reported in equine bone marrow derived MSCs (Violini *et al.* 2009) and umbilical cord matrix derived MSCs (Cremonesi *et al.* 2011). The expressions of these three pluripotency marker by isolated MSCs (UCB-WJ and AF) in the present study reflect the fact that equine fetal adnexa MSCs are more progenitor cells (Reed and Johnson 2008).

To summarize, our results suggest that equine AF contains higher number of viable MSCs with greater *in vitro* proliferation and differentiation capacities to that of UCB-MSCs, 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 studies. Further *in-vivo* studies will be required to substantiate the present study findings.

ACKNOWLEDGEMENTS

The authors are highly thankful the Dean, College of Veterinary and Animal Science, RAJUVAS, Bikaner; Director and In-charge, Equine Production Campus, National Research Centre on Equine, Bikaner, India for providing the necessary facilities and infrastructure to conduct the present study.

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