

Isolation and Characterization of Buffalo Wharton's Jelly Derived Mesenchymal Stem Cells

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Abstract

Bone marrow and adipose tissue are the most common sources of animal mesenchymal stem cells. However Wharton's jelly derived stem cells which could be collected non-invasively can be an alternative and good source of stem cells. The objective of present study was to isolate and characterize putative stem cell population from Wharton's jelly derived cells. Fibroblastoid, plastic adherent cells could be successfully isolated from Wharton's jelly and expanded *in vitro* conditions. Cells showed high proliferation ability and stained positively for alkaline phosphatase. Expression profile of surface cell markers and pluripotency markers were analyzed using immunocytochemistry and RT-PCR. Relative transcript abundance of pluripotency markers were analyzed by SYBR green based real time PCR. Karyotyping was done to demonstrate chromosome integrity. When corresponding induction conditions were applied, bWJ-MSCs differentiated into osteogenic, adipogenic and chondrogenic lineages which was confirmed through von Kossa, Oil Red O and Alcian blue staining respectively. In conclusion, buffalo Wharton's jelly represents a promising source of mesenchymal stem cells with high proliferative and differentiation potential and these cells could provide great scope for multiple regenerative therapies.

Keywords: Buffalo Wharton's jelly; Umbilical cord; Jelly explants; Immunocytochemistry; Differentiation

Introduction

Fetal and adult stem cells have the potential to play vital role in livestock production and management. Fetal stem cells in comparison to stem cells from other sources are easily accessible and can be isolated in larger quantities. Additionally it can provide new insights into development biology of animals. Fetal stem cells may develop as a valuable source of stem cells for clinical applications in large animals. According to ISCT (International Society for Cellular Therapy) mesenchymal stem cells can be defined by three criteria 1) Mesenchymal stem cells must be plastic-adherent when maintained *in vitro*; (2) Mesenchymal stem cells must express CD73, CD90 and CD105, and lack expression of CD34, CD45, CD14 or CD11b, CD79a or CD19 and MHC class II antigens; (3) The MS cells must differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* [1].

MSCs are most commonly sourced from bone marrow. As bone marrow harvesting is a highly invasive procedure, alternative sources for MSC isolation have been pursued. Recent studies reported MSCs can be sourced from several regions of umbilical cord including Wharton's jelly [2]. Wharton's jelly is the gelatinous connective tissue from umbilical cord and is composed of myofibroblast-like stromal cells, collagen fibers, and proteoglycans [3]. Wharton's jelly cells were successfully isolated from human and porcine umbilical cord tissue and explanted as primary culture [2,4]. Studies have reported that they possess the multilineage differentiation potential [5,6]. They have been isolated from different species like humans, dog, pig, horse, sheep, bovine and goat. The objective of the present study was to isolate, culture and characterize buffalo Wharton's jelly derived mesenchymal stem cells (bWJ-MSCs)

Materials and Methods

All the chemicals used in this study were procured from Sigma (St. Louis, MO, USA), unless otherwise indicated. The primary and secondary antibodies used for immunocytochemistry were purchased from Santa Cruz Biotechnology, USA.

Isolation and culture of Buffalo Wharton's jelly derived stem cells

Gravid buffalo uteri (n=4) at later gestation period ~3 M old were collected from local abattoir and transported within two hours to the Reproductive Physiology Laboratory of Physiology and Climatology Division, Indian Veterinary Research Institute, Izatnagar, India. The umbilical cords were processed for isolation of WJ-MSCs as previously described by Babaei et al. [7], with some modifications. Briefly, after rinsing in normal saline, the cords were aseptically stored at 4°C in sterile saline until processing. The umbilical cord vessels were removed manually from cord segments, and the exposed mesenchymal connective tissues were cut into very small pieces or explants, approx. 1–2mm, before placing them in a disposable 35 mm tissue culture dish. The explants were cultured in DMEM medium fortified with 15% fetal bovine serum, 200IU/ml penicillin, and 200 µg/mL streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO₂. Wharton's jelly fragments were observed to be attached within 72 h of plating, jelly explants were removed from culture dishes a week after plating and the expanded cells were cultured for at least 7 more days and the medium was refreshed at every third day. Two weeks later the attached cells were harvested by 5 minutes enzymatic digestion where, cells were detached by treating with accutase (Sigma) for passage. For all the experiments stem cells were taken from third passage.

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Received March 24, 2014; Accepted May 14, 2014; Published May 16, 2014

Citation: Sreekumar TR, Ansari MM, Chandra V, G Sharma T (2014) Isolation and Characterization of Buffalo Wharton's Jelly Derived Mesenchymal Stem Cells. J Stem Cell Res Ther 4: 207. doi:10.4172/2157-7633.1000207

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Alkaline phosphatase staining

Third passage bWJ-MSCs cultured and which formed a monolayer were subjected to alkaline phosphatase staining. Briefly, cell monolayer was rinsed in DPBS, fixed in 4% paraformaldehyde and then overlaid with 25 mM Tris HCl (Promega, USA), 150 mM NaCl containing 8 mM MgCl₂, Naphthol AS-MX Phosphate (0.4 mg/ml) and Fast Red TR salt (1 mg/ml) (Millipore, USA) for 20-30 min at 37°C, rinsed again with DPBS. Similarly treated buffalo fetal fibroblast monolayer acted as negative control. Stained cells were observed under inverted light microscope (Olympus-CKX-41, Japan) [8].

Osteogenic differentiation

Cells of third passage which formed confluent monolayer were used to induce osteogenic differentiation. Cells were cultured in DMEM medium supplemented with 50 mg/ml ascorbic 2-phosphate, 10 nM dexamethasone and 10 mM β-glycerophosphate for three weeks; von Kossa staining was then used to observe matrix mineralization. For staining, the cultures were first fixed in 70% methanol for 10 min and then subjected to von Kossa staining as per standard protocol [9].

Adipogenic differentiation

For adipogenesis, DMEM medium containing 100 nM dexamethasone and 50 mg/ml indomethacin were added to 60–80% confluent culture of passage-3 stem cells. Three weeks later, cells were fixed with 4% formalin at room temperature, washed with 70% ethanol and stained by Oil Red O solution in 99% isopropanol for 15 min [10].

Chondrogenic differentiation

Chondrogenic differentiation was induced in confluent monolayer cultures of bWJ-MSCs using a specific chondrogenesis differentiation kit (StemPro, Invitrogen). Differentiated cells were stained with Alcian blue 8GX (Sigma-Aldrich) after 16-20 days [11].

Karyotyping

After 72 hours of growth, cells of 3rd passage were incubated in 0.4 μg/ml colchicine for 2 hrs at 37°C in 5% CO₂ incubator. Cells were washed twice in PBS and harvested using accutase. The cells were later suspended in hypotonic solution (75mM KCl) for 20 minutes at 37°C. The cells were then fixed in chilled cornoys fixative (3:1 methanol/glacial acetic acid) for 5 minutes at room temperature and centrifuged at 1400 rpm for 5 minutes. Then pellet was washed twice with fresh chilled fixative and metaphase spread was prepared by dropping the cells on pre cleaned ice cold glass slides. Slides were stained in 4% Giemsa solution for 12 minutes and observed under compound microscope using oil immersion at 100X.

Total RNA extraction and reverse transcription

Total RNA was isolated from third passage bWJ-MSCs cells using Tri Reagent RT (Molecular Research Center Inc., USA) according to manufacturer's recommendations. The harvested cells were

resuspended in 1 ml Tri Reagent RT, pipetted well and supplemented with 50 μl of bromoanisole followed by gentle shaking for 15 s. The mix was centrifuged at 12000 rpm for 15 min at 4°C. The upper aqueous phase containing RNA was transferred to a new 1.5 ml eppendorf tube and the RNA was precipitated by mixing 0.5 ml of isopropanol. RNA was precipitated as a white pellet on centrifuging at 12000 rpm for 5 min at 4°C. The pellet was once again washed with 75% cold ethanol, air dried and dissolved in RNase-free water and stored at -80°C until used. The quality and concentration of RNA was assessed by Nano-drop (Thermo Scientific, USA). Reverse transcription was carried out using Revert aid kit (Fermentas, USA) as per manufacturer's instructions. Briefly, cDNA was synthesized in a total of 20 μl reaction volume, using 2 μg of RNA isolated from cAF-MSCs. Reaction was reverse-transcribed using Molony-Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (Fermentas, MD, USA) by incubating at 70°C for 5 min followed by incubation at 25°C for 5 min, 42°C for 60 min and finally reaction was stopped by incubating for 10 min at 70°C. The quality of cDNA was assessed by an amplification reaction for a housekeeping gene β-actin. RT-PCR analysis of surface markers was done for CD90, CD 73 and CD 105 using primers details of which given in Table 1.

Relative expression level of pluripotency markers (Oct4, Sox2 and Nanog)

Oct4, Sox2 and Nanog were analyzed using SYBR Green based real time assay with 2 μg of initial template in each qPCR reaction (MX3000P; Stratagene, CA, USA). Each reaction was prepared using Maxima™ SYBR Green/ROX qPCR master mix (2X) (Fermentas, MD, USA). The qPCR reaction was optimized prior to actual experimentation with regard to primer concentrations, reaction efficiency and denaturation/extension temperature for each pair of primers. The optimized reaction was carried out in duplicates at a final reaction volume of 25 μl containing 1 μl (0.5 μmol/L concentrations) of each forward and reverse primer, 3 μl of cDNA, 7.5 μl of nuclease free water and 12.5 μl SYBR Green qPCR master mix (2X). No-reverse transcription control (No-RT) and No-template controls (NTC) were also included in each qPCR reaction, whereas β-actin was used as the endogenous control. The qPCR cycling was performed as follows; initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 25 s, annealing at 55-60°C for 20 s varied with respect to primers used as given in Table 2 and extension at 72°C for 25 s and finally a melting curve profile was set at 95°C (1 min), 60°C (1 min), 95°C (30 s). The final dissociation stage was performed for fluorescent acquisition and reaction specificity. The cycle threshold (Ct) values given by the MxPro software (Agilent Technologies, USA) were used for further analysis. Relative expression level of pluripotency markers were analyzed using 2-ΔΔCt method and the results were depicted graphically [12].

Immunocytochemistry

Third passage bWJ-MSCs cultured in 24-well plates up to 80% confluency were fixed using 4% paraformaldehyde, permeabilized

Target genes	Primer sequences, 5'-3'	Gene bank accession no	Annealing temperature	Product size (kb)
CD 73	S: CTGAGACACCCGGATGAGAT A: ACTGGACCAGGTCAAAGGTG	BT_02624	55	160
CD 90	S: GTGAACCAGAGCCTTCGTCT A: GGTGGTGAAGTTGGACAGGT	NM_001034765.1	55	201
CD 105	S: ACAAAAGCCTCGTCCTACCT A: TGTGGTTGGTCTACTGCTC	NM_001076397.1	59	177
β actin	STEM TAG PCR primer set Cell bio labs inc		55	172

Table 1: Oligonucleotide primers used for RT-PCR.

Target genes	Primer sequences, 5'-3'	Gene bank accession no	Annealing temperature	Product size (kb)
Oct4	S: GAGCCGAACCCTGAGGAG A: AGGGTAAGCCCCACATCG	JF898834.1	60	125
Sox	S: CTATGACCAGCTCGCAGAC A: ACTTCACCACCGAGCCCA	GQ451841.1	60	111
Nanog	S: GCAGGTGAA GACCTG GTTC A: CCACATGGGCAGGTT TCCA	JF 898835.1	60	175
β actin	S: AGA TTG GCA TGG CTT TAT TTG T A: CTG TAG AAC TTT GGG AAT GCT C	BT030480	51	143

Table 2: Primers used for Real time PCR.

with 0.1% Triton-X-100 (only for transcriptional factor localization) and incubated with 0.01 M sodium citrate solution for 10 min at room temperature to expose the antigens. Permeabilized cells were washed in DPBS, followed by blocking the background staining by incubating with normal donkey serum (Santa Cruz, USA; #sc-2044) equilibrated in 0.5 M DPBS (pH 7.4) for 30 min. The cells were overlaid with primary antibodies at a dilution rate of 1:200 and incubated for 1 h at 37°C. After brief washing with DPBS, the cell monolayer was incubated with Fluorescein isothiocyanate (FITC) conjugated donkey anti-goat secondary antibody at 1:400 dilution rate for 45 min at 37°C in dark. Thereafter, the cells were washed with DPBS and observed under the fluorescence microscope. Negative controls were exempted from exposure to primary antibody. Primary antibodies used in this experiment were against CD-73 (#sc-32299), CD-90 (#sc-59396), CD-105 (#sc-18838), CD-34 (#sc-7324), Oct-4 (#sc-8628), Nanog (#sc-30328), Sox-2 (#sc-17320).

Statistical analysis

Real time PCR data was analyzed by one way ANOVA using graph pad prism (La Jolla, CA, USA) at 0.05% level of significance. Differences with $p < 0.005$ were considered as statistically significant.

Results

Growth and culture characteristics of cWJ-MSCs

Morphologically cells appeared as migrating from jelly explants during primary culture incubation (Figures 1A and 1B) Later its morphology changed to typical fibroblast like spindle shaped cells (Figure 1C). After 7 days of incubation plastic adherent primary culture of monolayer cells with 80% confluent monolayer was

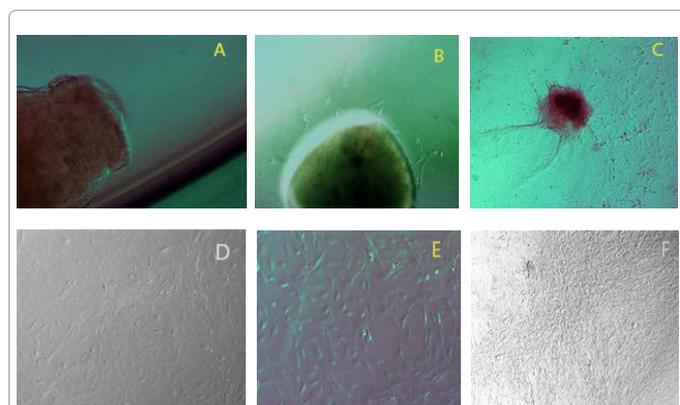


Figure 1: Morphological features of bWJ-MSC culture. Primary culture of bWJ-MSCs (A) On the day of seeding of jelly explant. (B) On day 3 attached jelly explant. (C) On day 5 fibroblastoid shaped cells extruding from jelly explant. (D) & (E) On day 7 typical fibroblast like spindle shaped cell morphology. (F) Confluent cells after first passage.

obtained (Figure 1D and 1E). Passaging of cells was done with accutase treatment and further re-seeding and culture was done (Figure 1F). Cell monolayer formed by buffalo Wharton's jelly derived MSCs after 3rd passage showed alkaline phosphatase activity on day four of culture, as it appeared red when visualized under inverted microscope demonstrating the undifferentiated nature of these cells (Figures 2A and 2B).

Differentiation potential of bWJ-MSCs

In osteogenic cultures, cells proliferated and reached to complete confluency after 6-8 days of incubation. The cellular aggregates were then observed in osteogenic differentiation culture plates by 7-8th day of co-incubation and gradually increased till the end of the experiment. These aggregates were characterized by calcium deposits, which were demonstrated brownish black through von Kossa staining (Figure 3C). Small lipid droplets within the cytoplasm of bWJ-MSCs started appearing by 12th day of co-culture with specific induction media. Reddish colored lipid droplets could be demonstrated by Oil Red O staining on 21st day of culture (Figure 3B). The chondrogenic potential of bWJ-MSCs was evaluated by in vitro culture of these cells in a serum-free chondrogenic specific medium. After 3 weeks of differentiation, the accumulation of sulfated proteoglycans was visualized by alcian blue staining (Figure 3A).

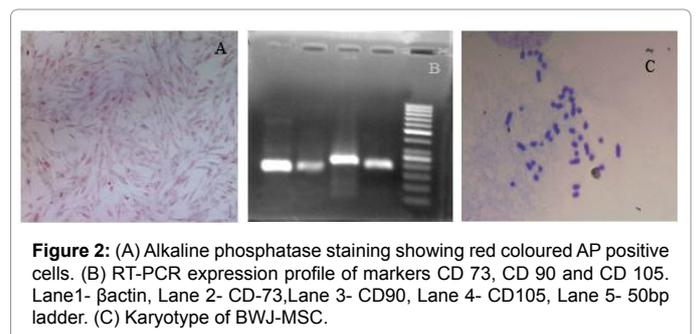


Figure 2: (A) Alkaline phosphatase staining showing red coloured AP positive cells. (B) RT-PCR expression profile of markers CD 73, CD 90 and CD 105. Lane 1- βactin, Lane 2- CD-73, Lane 3- CD90, Lane 4- CD105, Lane 5- 50bp ladder. (C) Karyotype of BWJ-MSC.

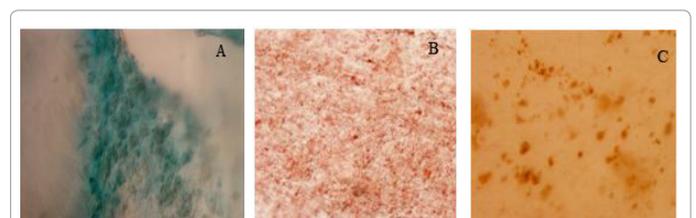


Figure 3: *In vitro* multilineage differentiation of bWJ-MSCs. (A) Positive staining of Alcian blue indicated invitro expanded bWJ-MSCs could differentiate into chondrocytes. (B) Specific Oil red O staining indicated adipogenesis induced lipid droplets observed in red colour on incubation with specific adipogenic induction media. (C) Brownish black coloured mineral deposition as demonstrated by Von kossa staining showing osteogenic differentiation.

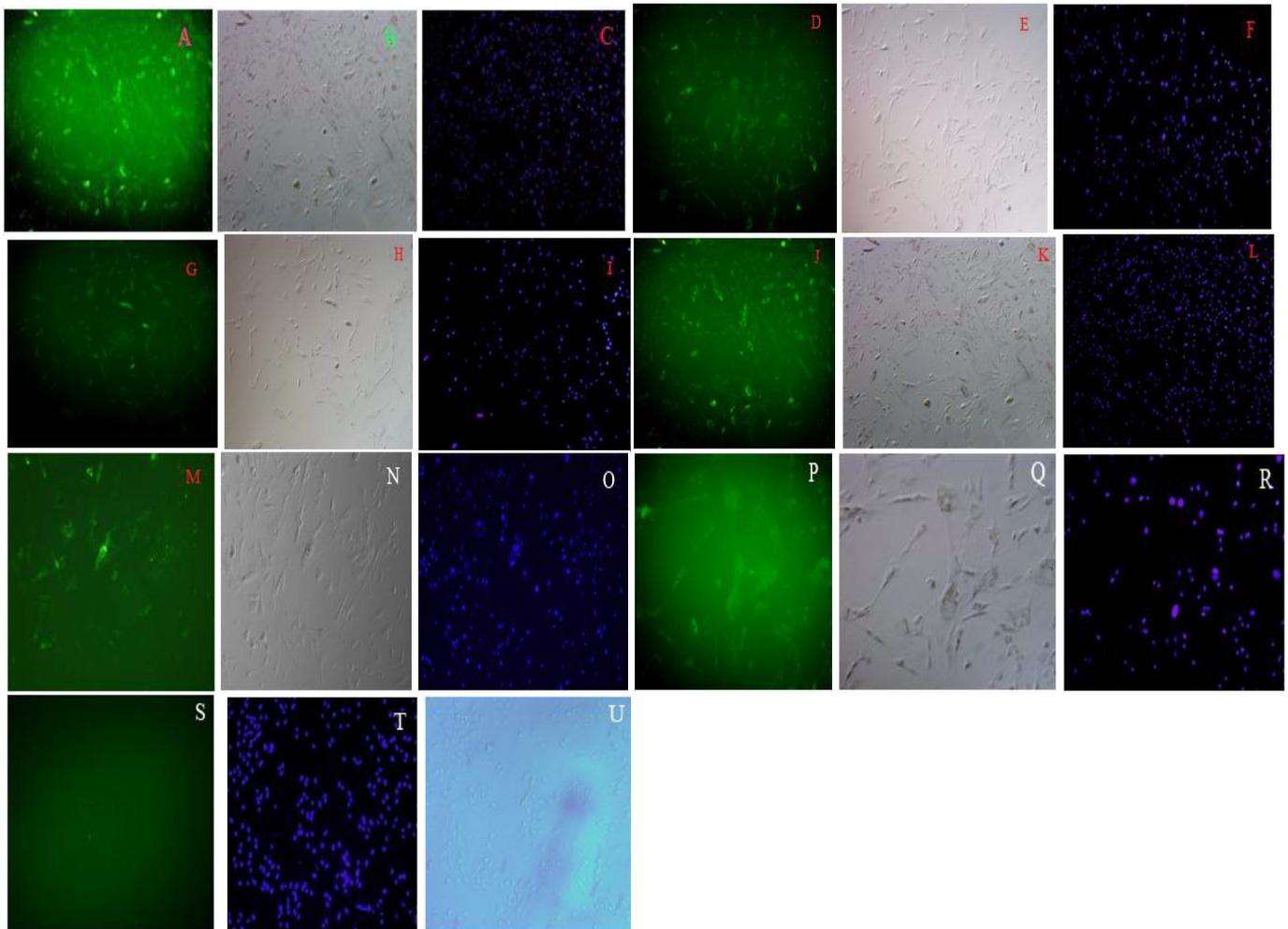


Figure 4: Immunocytochemistry. Immunolocalization of both surface antigens and pluripotency associated markers in bWJ-MSCs monolayer. Cells were stained with primary antibodies directed against (A) CD73, (D) CD90, (G) CD105, (J) Oct4, (M) Sox2, and (P) Nanog, (S) CD34 and stained with FITC conjugated secondary antibodies. In panels (B, E, H, K, N, Q, U) representative fields as observed under bright field. In panels (C, F, I, L, O, R, T) fluorescent blue coloured DAPI stained representative fields.

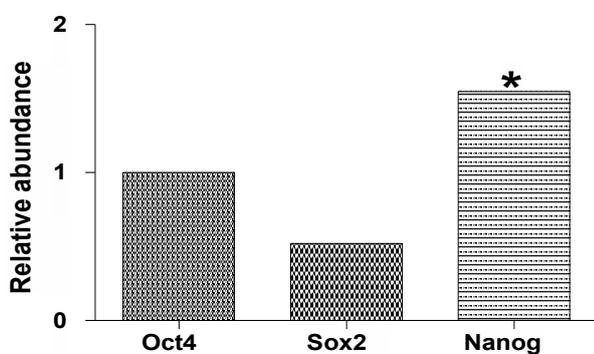


Figure 5: Real time PCR analysis of pluripotency markers in expanded bWJ-MSCs. Bar diagram showing higher relative abundance (mean \pm SE; n=2) of Nanog transcripts in comparison to Oct4 and Sox2. Asterisk(*) indicates significant difference ($P < 0.005$).

Karyotyping

Karyotype of bWJ-MSCs showed intact chromosomes. Metaphase spreads of buffalo WJ- MSCs showed normal karyotype at different passage and there were no abnormalities like chromosomal fragmentation (Figure 2C).

Surface antigen profile of bWJ-MSCs

A group of positive surface markers for MSCs viz. CD73, CD90, CD105 and Stro1 were tested by RT-PCR and immunocytochemistry. bWJ-MSCs were found positive for CD-73, CD-105 and CD-90 (Figure 4A-4I) but negative for haematopoietic cell surface marker CD-34 (Figure 4S-4U).

Expression of pluripotency associated markers in bWJ-MSCs

Pluripotency associated transcriptional factors (Oct-4, Nanog, Sox-2) were successfully localized in the bWJ-MSCs cell monolayer via immunocytochemistry (Figure 4J-4R) and Real time PCR (Figure 5)

The transcript copy number of Nanog was found relatively higher than that of Oct4 and Sox2.

Discussion

Considering the invasive techniques involved in isolation of MSCs there is an increasing interest in investigating the potential of MSCs in adult and extra-embryonic sources, such as fetal membranes, amniotic fluid and umbilical cord matrix [13-15]. Also the collection of extra-embryonic adnexa is safe and easy, are viable, accessible and not risky source of MSCs. High initial sample volumes Wharton's jelly could easily be collected at the delivery without any invasiveness for the buffalo.

The Wharton's Jelly, connective tissue of the umbilical cord has recently drawn an attention of researchers to focus as a new potential source of MSCs both in humans and farm animals [16-20].

In the case of buffalo, Wharton's jelly derived MSCs isolated by explant culture method, homogenous fibroblastoid cells colonies could be established successfully. Similar reports exist from Babei et al. [7] and later by Azari et al. [21], Pratheesh et al. [22] for caprine species.

The property of plastic adherence itself is not sufficient to allow for the purification of MSCs. Much valuable information can also be gained from a systematic analysis of cell surface molecules on MSCs. For characterization, we carried out alkaline phosphatase staining and immunophenotyping assays, as adapted by Hoynowski et al. [23]. AP positive staining demonstrated their high phosphatase activity – a unique feature of undifferentiated stem cells. STRO-1 was identified as an antibody that reacted with non-haematopoietic progenitor bone marrow stromal cells [24]. Endoglin (CD105) [25] antibody has been used in immunomagnetic selection methods for human MSCs, although CD105 is dominantly associated with endothelial cells [26]. Wharton's jelly derived stem cells exhibited specific mesenchymal stem cell marker expression (CD73, STRO-1, CD90 and CD105) based on RT-PCR and immunocytochemistry results.

The general strategy for identifying in vitro cultivated mesenchymal stem cells as per ISCT (International Society for Cytotherapy) is to analyze the expressions of cell-surface markers such as CD-73, CD-90 and CD-105 and lack expression of CD34 or CD19 [1,27-29]. We demonstrated that the bWJ-MSCs are positive for CD-73, CD-90, and CD-105 whereas, negative for CD-34, a cell-surface marker associated with lympho-hematopoietic cells. They were also found positive for pluripotency markers like Oct4, Sox2 and Nanog. The real time data demonstrated higher levels of Nanog marker expression in comparison to other pluripotency markers.

Present study demonstrated that they successfully differentiated into adipogenic, osteogenic and adipogenic lineages. This is at par with similar reports from human, equine, and bovine umbilical cord tissue and explanted as primary culture [4,23,30].

Conclusion

Present study showed that buffalo mesenchymal stem cells could be successfully isolated and cultured and characterized from buffalo Wharton's jelly. Their morphology, immunophenotype and differentiation potential are comparable with MSCs from other source. Expression of pluripotency markers demonstrates their potency. Data from the present study demonstrates the proliferative and differentiation potential of bWJ-MSCs and Buffalo Wharton's jelly could serve as a potent source of mesenchymal stem cells. With future

research and standardization they could serve as valuable resource for various clinical applications.

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