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Expression of early transcription factors by mesenchymal stem cells derived from ovine umbilical cord Wharton's jelly

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into mesodermal lineages. Despite major progress in our general knowledge related to the application of adult stem cells, finding alternative sources for bone marrow MSCs has remained to be challenged. In this study, we utilized ovine umbilical cord Wharton's jelly as a primary source for isolation of MSCs since it is a rich source of MSCs and no ethical issues were involved. Ovine umbilical cord Wharton's jelly segments were digested enzymatically and cultured *in vitro* in culture medium. In addition to the study of their morphology and colony forming units, the expression of pluripotent stem cell markers by the isolated MSCs were also studied. The MSCs were plastic adherent, clonogenic and their morphology were polygonal, star shaped and fibroblast like. They revealed a strong expression of pluripotent stemness markers Oct4, Sox2, Nanog and Alkaline phosphatase. These cells confirmed their ability of self-renewal by expressing Sox2 gene and their properties of pluripotency and plasticity by expressing Oct4, Nanog and Alkaline phosphatase. The study revealed that Wharton's jelly is a rich source of stem cells with stemness properties and mesenchymal like morphology and could be used as an alternate for the bone marrow derived MSCs for cell based regenerative therapies.

Key words: Mesenchymal stem cells, Pluripotent marker genes, Sheep, Wharton's jelly

Mesenchymal stromal cells (MSCs) have multipotent properties suitable for tissue engineering and regenerative medical applications. The main factors required for a cell to be regarded as MSC are— their adhesion potential in monolayer culture during *in vitro* conditions, ability to maintain their undifferentiating characteristics during extended passaging, and multilineage differentiating potential (Rentsch *et al.* 2010).These adult stem cells are attractive candidates for cell based therapies because of their easy isolation, intrinsic ability to self bio-preserved with minimal loss of potency, multilineage differentiation and amenability to allogenic vs autologous MSCs transplants (Kolf *et al.* 2007, Zuk *et al.* 2001). In the ensuing decades, extensive research has been carried out to unlock the therapeutic potential of MSCs. Although bone marrow provides a universal source of MSCs, owing to certain shortcomings of obtaining the MSCs like pain, low cell number upon harvesting, high degree of viral contamination, decrease in the proliferative/differentiating capacity along with age, alternative sources were sought and subjected to intensive investigation (Huang *et al.* 2009, Heidari *et al.* 2013). Pluripotency is a key measure of the performance of stem cells. There are number of ways of identifying the pluripotency but it was found that the best and accurate way of identification of this phenotype is by using specific molecular markers in these cells. The study of gene expression profiles is extremely valuable for identification of candidate stem cell genes and markers of different differentiation lineages. Studies showed that the expression of Oct4, Nanog and Sox2 indicated pluripotent nature of stem cells (Tai *et al.* 2005, Carlin *et al.* 2006, Yu *et al.* 2007). To date no investigations have been found about the isolation and *in vitro* cell culture of WJ-MSCs from ovine umbilical cord tissue Wharton's jelly (UCT-WJ).Therefore in this experiment, the isolation of MSCs from ovine UCT-WJ, their culture characteristics and some stemness marker gene expression were studied.

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MATERIALS AND METHODS

The work was conducted following approval of the Institutional Ethical Committee for Stem Cell Research, Tamil Nadu Veterinary and Animal Sciences University, Chennai. All the chemicals were procured commercially.

Generation and expansion of UCT- WJ derived MSCs: Ovine foetuses (mid gestation) were collected from the local slaughterhouse and transported to the laboratory in sterile phosphate buffered saline (PBS) supplemented with penicillin and streptomycin on ice. The umbilical cord was

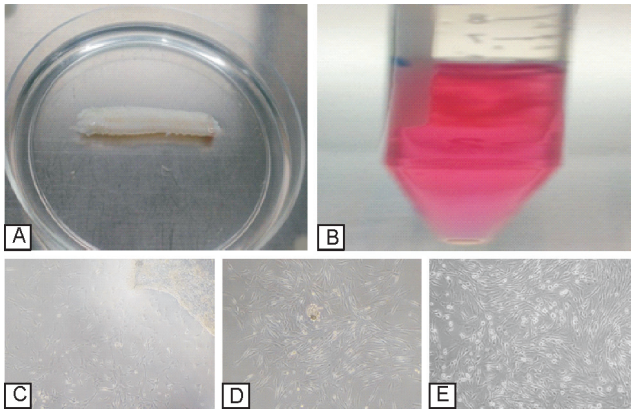

Fig. 1. (A-E). Isolation and primary culture of ovine UCT-WJ tissue derived MSCs. **A**. Harvested ovine umbilical cord tissue; **B**. enzymatic digestion of WJ. **C-E**. Culturing of WJ derived MSCs on 3rd, 6th and 8th day of primary culture. **C**. Irregular and heterogenous MSCs isolated from a digested fragment of WJ in primary culture plates; **D**. Proliferating irregular shaped MSCs; **E**. Plate adherent, homogenous, fibroblast like cells.

rinsed with sterile PBS for several times and cut into 2–4 cm lengths (Fig. 1A) and WJ was separated. Under laminar hood, the specimen was mechanically minced into small pieces and subjected to enzymatic digestion (Azandeh *et al.* 2012). Briefly, the WJ pieces were digested using enzyme cocktail containing 4 mg/ml of collagenase type I and 1 mg/ml of hyaluronidase for 1 h followed by 0.1% trypsin-EDTA for 30 min (Fig. 1B). At the end of this period, the floating cells were separated by centrifugation at 200 × g for 10 min and the cell pellet was suspended in culture medium composed of Dulbecco modified eagle's medium (DMEM) with 2 mM L-glutamine, 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin and cultured at a temperature of 37°C with 5% CO_2 until migration of MSCs. 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. Subculture was repeated till passage 5 until sufficient cells were provided for the next stage of experiments.

Morphology of MSCs: The isolated MSCs were observed for their morphology under Inverted phase contrast microscope and photographed. The cells were also stained with Giemsa stain and observed under light microscopy.

Clonogenicity evaluation/colony forming unit (CFU) assay: The WJ-MSCs cultured in culture medium with 10% FBS were seeded in 6 well tissue culture plates at a seeding density of 10 cells/cm^2. After 14 days, the cells were washed in DPBS, fixed in 4% paraformaldehyde for 20 min and stained with 0.5% Crystal violet stain for 5–10 min and rinsed in tap water. Colonies consisting of a minimal cell number of 50 cells were visualized under light microscope.

Expression of pluripotent marker genes by reverse transcriptase polymerase chain reaction (RT-PCR): Expression of pluripotency genes was analysed by RT-PCR as per the standard protocol. Briefly, the total RNA was extracted from the WJ-MSCs at passage 5 using RNeasy kit. 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. The PCR reactions was performed in a 25 µl reaction solution using Ampliqon red dye master mix (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 table1. Amplified DNA fragments were separated on 2% agarose gel containing 0.1 µg/µl ethidium bromide and visualized under UV light.

RESULTS AND DISCUSSION

In recent years, parallel to the great efforts for exploring the novel and alternative sources of stem cells in animals, the umbilical cord appeared to be a promising reservoir of fetal cells that could be easily used as multipotent stem cells. In this study, MSCs were successfully isolated from ovine UCT-WJ and expanded in mixed explant-enzymatic culture method. We isolated 10–15 × 10^4 cells (range is 10–30 × 10^4) from primary culture. The cultures were routinely visualized under inverted phase contrast

Table 1. Sequence of primers used for expression of stem cell markers

Molecular marker	Forward primer	Reverse primer	Annealing temperature (°C)	Product size (bp)
(Sox 2)	5'-AACCAAGACGCTCATGAAGAA-3'	5'-GTACTGCAGGGCGCTCAC-3'	61	277
(Oct 4)	5'-ACCCAGGCTGATGTGGGGCTC-3'	5'-TGTGGCTAATTTGCTGCAGGGTG-3'	66	314
Nanog	5'-GCCCCTTAGTAAGCTGCTTTT-3'	5'-GGGGTGGTGGAAATCAGTAA-3'	58	317
Alkaline phosphatase	5'-ACCAATGGCAACCTGCTGTA-3'	5'-CTCCTCCAGGATCTTGGCTA-3'	59	198
β actin	5'-AGGAGGGAAGGCTGGAAGAG-3'	5'-GAAATCGTCCGTGACATCAA-3'	59	182

microscope. The cells started sprouting on 24–48 h itself in *in vitro* cultures, grew more rapidly and reached 80–90% confluency on eighth day of culture. The primary cells isolated were heterogeneous, with mesenchymal like cells possessing long processes as well as small round cells with a high nuclear to cytoplasm ratio. Through 10 passages, the populations were morphologically similar to the parent cells (Fig. 1C-E). The isolated MSCs maintained their morphology and as well as their multiplication characteristics. The cells stained with 0.1% Giemsa stain and showed a varying morphology with spindle shaped, elongated, cuboidal and fibroblast like cells (Fig. 2A). Azandeh *et al*. (2012) reported epitheliod or short fibroblast like cells in early passages which then transformed into long spindle shaped fibroblast like cells in further passages. Similar cell morphology was observed by Moshrefi *et al.* (2010) in MSCs isolated from caprine umbilical cord matrix and Grzesiak *et al.* (2011) in ovine adipose derived MSCs.

The ability to generate clones is a formal demonstration of the self-renewal ability, a characteristic of stem cells

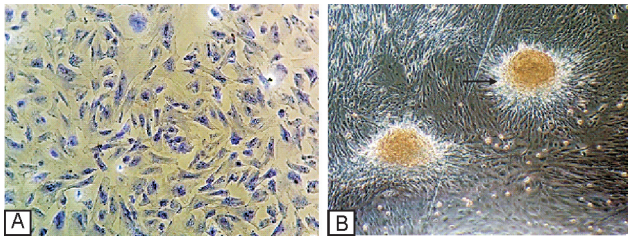

Fig. 2 (A-B). **A.** Wharton's jelly derived spindle shaped MSCs showing large nucleus with extended cytoplasm stained with Giemsa stain (100×); **B.** MSCs isolated from WJ explants form colonies (arrow mark) of tightly packed small round cells after 14 days of culture (100×).

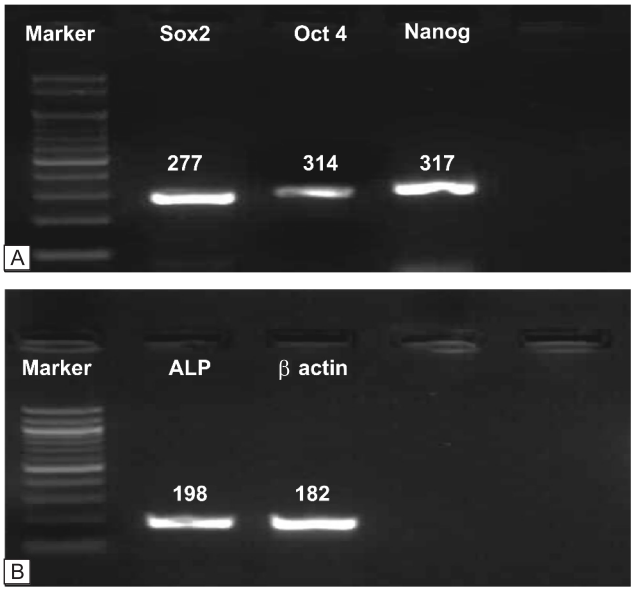

Fig. 3 (A-B). Agarose gel electrophoresis of analysis of RT-PCR product for the pluripotent molecular markers: **A**. Sox2, Oct4, Nanog; **B**. Alkaline phosphatase and endogenous control β actin with the respective products in ovine WJ derived MSCs.

populations (La Racco *et al.* 2009). To give a formal demonstration of the self-renewal capability of WJ-MSCs, the presence of clones was assessed in culture dishes under light microscopy. In this study, we demonstrated that the isolated MSCs have clonogenic properties (Fig. 2B). Similar results were observed in caprine (Moshrefi *et al.* 2010), human (Venugopal *et al.* 2011), UCT-WJ derived MSCs.

The cultured MSCs isolated from WJ were tested for their stemness property by detecting the expression of pluripotent marker genes. In this study, the stemness and early lineage specific marker expression profile of Oct4, Sox2, Nanog and alkaline phosphatase genes were analysed from WJ derived MSCs. The RT-PCR analysis of cultured cells at fifth passage indicated positive expression of these genes (Fig. 3 A,B). The gene specific bands were purified and the resulting sequences were aligned and analysed for confirmation. A number of transcription factors like Oct4, Sox2, Nanog and alkaline phosphatase, cMyc and Klf_4 play a critical role in maintaining pluripotency of stem cells have now been identified and their expression was used to characterize stem cells in various species (Niwa 2007).The Oct4 gene was proposed as a master regulator of pluripotency of the stem cells (Campbell *et al.* 2007). In this study, the RT-PCR analysis of expression of Oct4, Sox2, Nanog and alkaline phosphatase genes in the ovine UCT-WJ derived MSCs showed a positive expression of these genes. Our results are in agreement with the results of Singh *et al.* (2013) and Sreekumar *et al.* (2014) who found expression of these genes in buffalo umbilical cord matrix cells. Carlin *et al.* (2006) also reported expression of these genes in porcine umbilical cord matrix cells. The expression of these genes was also reported in MSCs derived from bovine UCT-WJ (Cardoso *et al.* 2012), caprine UCT matrix (Babaei *et al.* 2008, Moshrefi *et al.* 2010). Expression of early transcription factors were also observed in MSCs derived from visceral and subcutatneous adipose tissue in human (Potdar and Sutar 2010), sheep bone marrow and adipose tissue (Heidari *et al.* 2012), canine umbilical cord (Uranio *et al.* 2014), bovine umbilical cord blood (Raoufi *et al.* 2011), buffalo amniotic fluid (Dev *et al.* 2011), and buffalo fetal skin derived fibroblast cells (Yadav *et al.* 2012).

In conclusion, the present study indicated that MSCs can easily be isolated from the ovine UCT-WJ, expanded *in vitro* maintaining their MSC morphology. The strong expression of pluripotency marker genes provides evidence that these cells have stem cell characteristics and can be a potential source of MSCs for cell based therapies.

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