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Isolation and culture of putative mesenchymal stem cells from equine umbilical cord Wharton's jelly

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ABSTRACT

Despite major progress and knowledge related to the application of adult stem cells, finding alternative sources for bone marrow MSCs has remained a challenge in both humans and animals. In the current study, two protocols namely sequential enzymatic tissue digestion and tissue explant techniques were tried for successful establishment of MSC culture. Umbilical tissues were isolated each time of foaling from five sequential foalings of Marwari mares. Total cell yield, their growth potential and cryopreservation potential were studied. Adherent cell colonies could be established using both isolation methods. Both the cell populations yielded from different protocols performed similarly in terms of population doubling and CFU number value. Additionally, the cells proliferated vigourously and displayed a similar morphology of mesenchymal stem cells. The MSCs were plastic adherent, colonogenic and their morphology was polygonal and fibroblast like. During the proliferation, the cells exhibited density dependent inhibition; analysis of microbial contamination from bacteria, mycoplasma and fungi were negative; the population doubling time of the MSCs isolated was 34.8 h and 40.2 h in enzymatic treatment and tissue explant methods respectively, and diploid chromosome number of the cells was 64, and the diploid frequency was higher than 80%. In conclusion, this study reveals that both the techniques proved to be non-invasive, efficient, simple and quick for isolation and establishment of MSC culture of extra embryonic tissues from equines.

Key words: Collagenase, Equine, Mesenchymal stem cells, Tissue explants, Trypsin, Umbilical cord, Wharton's jelly

Stem cells represent the most promising cell types for cell therapy and have drawn considerable attention in equine veterinary medicine during the current past. This interest is mainly raised by the fact that the musculoskeletal system represents a major part of the horse's demand and value for sports and breeding (Koerner *et al.* 2006). 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 and Hui 2006). 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 (Vidal *et al*. 2012, Kisiday *et al.* 2008), 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 (Kasashima *et al*. 2011, Toupadakis *et al.* 2010). 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 also possess multilineage differentiation potential (D'Ippolito *et al.* 1999). Umbilical cord could represent an ideal source of MSCs other than bone marrow, considering the expression of pluripotency characteristics of these cells and the fact that it is easy to collect the sample from the umbilical cord in a non-invasive manner and the placenta including the cord is categorized as classified waste (Vidal *et al*. 2007). MSCs can be isolated from these tissues either by sequential enzymatic digestion or by tissue explant techniques. Many

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studies have reported the side effects of enzymes, endotoxin and chemotactic tissue breakdown products on the phenotype and behaviour of cells (Dragoo *et al*. 2003). Disadvantage in tissue explant technique is that only cells located at the tissue margin can migrate out of the tissue, so that not all MSCs residing in the tissue can be collected when using this technique. However, the different preparation techniques may potentially influence the growth characteristics and expression properties of the isolated MSCs. Therefore, the present study was conducted with an aim to isolate the MSCs using both the procedures and investigate and compare the growth patterns, colony forming units of the MSCs isolated from both the protocols used for the isolation of MSCs from equine umbilical cord Wharton's jelly.

MATERIALS AND METHODS

The present work was carried out at Equine Production Campus, ICAR-NRC on Equines, 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 (USA) and the plasticware from Eppendorf (Roskilde, Denmark) unless mentioned otherwise.

Sample collection: The samples (5) were obtained immediately after foaling from Marwari breed mares at Equine Production Campus, ICAR-NRC on Equines, Bikaner, Rajasthan. To prevent the contamination and damage to the tissues, the samples were collected immediately after foaling using sterilized syringes, surgical gloves and equipment (Fig.1). The umbilical cord Wharton jelly's samples were washed twice in 5% betadine solution, then with 70% alcohol; kept at 4°C in Dulbecco's Phosphate Buffer Solution (DPBS) with antifungal and antibiotic medium and were processed within 5 h.

Sequential enzymatic digestion technique: In sequential enzymatic digestion, the tissue pieces were made in to fine small pieces and each sample was diluted 1:1 with DPBS containing 100 IU/ml penicillin and 100 mg/ml streptomycin and to which 1 ml/1 g sample of a digestion solution (2% trypsin and 0.1% (w/v) collagenase type I) was added 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 1,000 rpm for 4 min at 4°C in a refrigerated centrifuge to obtain cell pellet. The supernatant was discarded and the cell pellet was resuspended in 1 ml of culture media containing Dulbecco Modified Eagle's Medium 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 fungal contamination of the cultures. The cell number was counted using haemocytometer. Cells were incubated at 37.5°C in a 5% CO_2 atmosphere and the culture medium was changed twice a week. Primary cultures (P0) were passaged using EDTA-Trypsin at confluency of the dish.

Tissue explant technique: In this technique, the Wharton's Jelly tissue was cut into very small pieces and dissected into pieces of approximately 0.25×0.25 cm size using a sterile surgical scalpel and forceps and then washed twice in DPBS. Tissue pieces were carefully placed into T25 cell culture flask and allowed to stick down to the plastic. After ensuring the settlement of the tissues, minimum 1 ml of above mentioned culture media was added initially for 5–6 h till the firm attachment of the tissues on to the plastic. Later, rest of 3 ml of culture media was added. The flask was further observed for cell migration from the adhered tissue explant and cell growth from time to time. Culture conditions were identical to those followed for the enzymatic digestion.

Cryopreservability of cells and recovery: The MSCs obtained through both the protocols were subjected for cryopreservation after passage 4. Briefly, the cells in the flask were initially washed two times with DPBS and EDTA-trypsin was added to remove the plastic adherent cells. The flask was treated on 37°C for 3 min and observed under the microscope for the cell removal from the plastic. After ensuring the cell removal, 4 ml of DPBS was added and the contents were emptied in a 15 ml tube and centrifuged for 4 min at 1,000 rpm. Meanwhile, the cryopreservation media using Dimethyl Sulfoxide (DMSO) (10% of the culture media) was prepared and kept ready. The cell pellet obtained after removal of the supernatant was mixed with 1 ml of cryopreservation media (DMSO

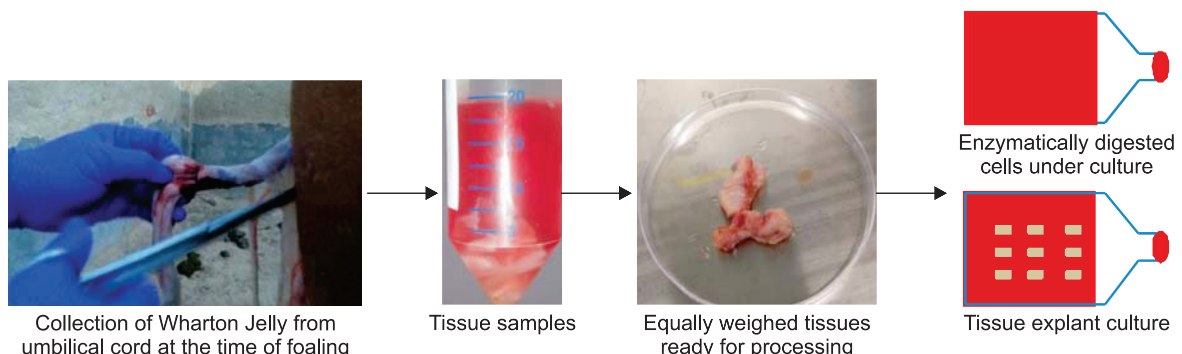


Fig. 1. Overall view of the work done for the isolation and culture of MSC from equine umbilical cord Wharton's jelly.

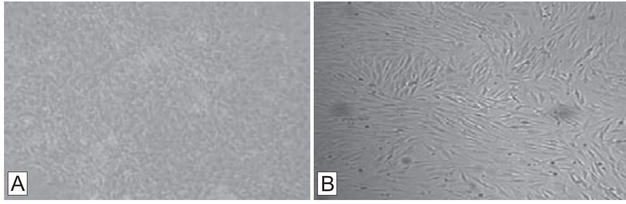


Fig. 2. Confluent cells. **A**. Enzymatic digestion **B**. Tissue explant technique.

and culture media) and were initially stored on –80°C and shifted to LN_2 storage cryocans on next day.

Cryovials were thawed in a 37°C water bath for 2 min till the liquid portion appears in the cryovial. The cells were immediately transferred to 5 ml of equilibrated expansion media and gently vortexed. The cells were centrifuged at 1,000 rpm for 4 min at room temperature. The cell pellet was resuspended in the culture media. The cell counts were made later on to see the effect of cryopreservation on the viability and cell number of the MSCs isolated from the both the protocols.

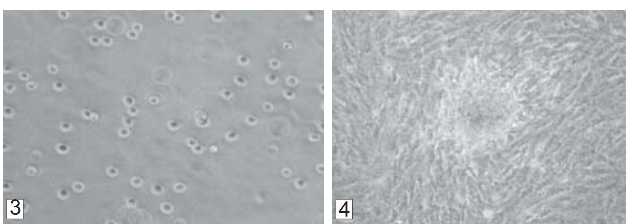
Viability assay: The cells isolated from the both techniques were used for determining the cell viability using trypan blue staining as described previously (Weingartl *et al.* 2002). The number of dead cells was determined from a field of 500–1,000 cells.

Colony forming unit (CFU) assay: 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 6 well plates and cultured in 5% CO_2 and 90% humidity at 37.5ºC 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 6-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).

Charcterisation of the MSCs: The MSCs isolated from the both the protocols were characterized for the pluripotency gene expression (OCT4) through RT-PCR and alkaline phosphatase (AP) staining (Talluri *et al*. 2015).

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 expressed as $P<0.05$ were considered statistically significant.



Figs 3–4. **3**. Cells after thawing. **4**. Colony forming units observed during the culture.

RESULTS AND DISCUSSION

Morphological assessment: Cells isolated out of both the protocols exhibited large and occasionally multi-nucleated cell types; and in both cultures, we observed small, spindle-shaped and mostly mono-nucleated cell types in the primary culture (Fig. 2). This heterogeneity cell population was no longer observed at the second passage as the smaller spindle-shaped fibroblastoid cells started to dominate and proliferate even after further passages. Individual spindle-shaped cells appeared after 3 to 4 days of initial culture, while colonies shown to pop up as early as 5 days post seeding (Fig. 4) and the first subculture was done 7 days after initial seeding with respect to the both the culture methods.

Growth curve analysis: The growth curve of cells isolated (from both the protocols) from equine umbilical cord Wharton jelly tissue had classic "S" shape involving a lag, log and plateau phases (Fig. 5). The population doubling time observed was approximately 38.6 h and 40.2 h for the enzymatic digested and tissue explanted cells (Fig. 6). 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.

Cell viability: Cells were harvested and frozen after 4 passages in both the cultures. The viability (expressed as mean±SD) of the cultures before freezing was 96.54±3.22%, 97.21±2.98 and 92.36±2.77%, 92.49±3.46 after thawing in enzymatic digested and tissue explanted cultures, and this difference was non-significant ($P>0.05$). These results showed that the cells were healthy under these culture conditions and that cryopreservation had little effect on the viability of the cells isolated using either of the techniques (Fig. 3). The cells were further cultured for their characterisation and expression of pluripotency.

CFU analysis: Colony forming units in both the cell cultures were analysed and listed (Tables 1, 2). There was no significant ($P>0.05$) difference observed in both the situations in respect of colony forming from the cells union.

Characterisation of the MSCs: The MSCs isolated from the both the protocols expressed the OCT4 in RT-PCR analysis and stained positive for the alkaline phosphatase staining *(Data not shown)*.

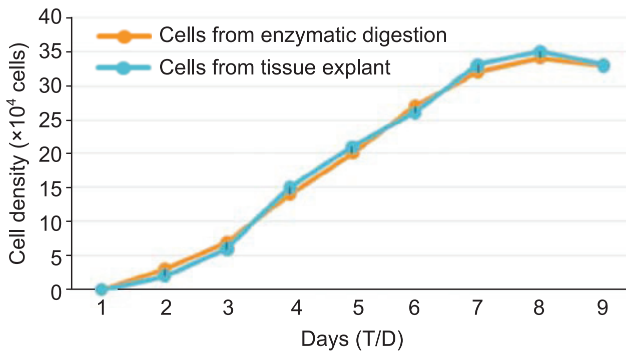


Fig. 5. Growth curve analysis of cells derived from both the protocols.

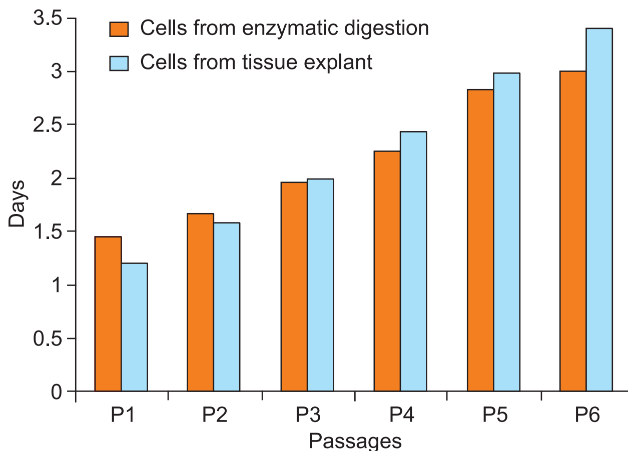


Fig. 6. Cell doubling time of the MSCs derived from both the protocols.

The MSCs of larger animals (sheep, dog and horse) are usually used for a preclinical evaluation of joint tissue regeneration (Dragoo *et al*. 2003). Though, bone marrow is a suitable source of MSCs, due to certain hurdles of obtaining the MSCs like pain involvement due to invasive procedure, low cell number upon seeding and harvesting, high degree of mycoplasma, fungi, bacterial and viral contamination, decrease in the differentiating ability along with age, alternative sources have been sought for this technique (Huang *et al.* 2009, Heidari *et al.* 2013). 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 equine umbilical cord Wharton's Jelly and expanded in both enzymatic digestion and tissue explant culture method. We

Table 1. CFU analysis in the MSC isolated through the enzymatic digestion technique

Density (cells/cm^2)	Total cells	CFU	1CFU each
100	850	1.5±0.21	566.66
250	2437	10.28±2.14	237.06
500	4657	21.64±1.69	215.2
1000	9600	30.64±2.97	313.31

Table. 2. CFU analysis in the MSC isolated through the tissue explant technique

Density (cells/cm^2)	Total cells	CFU	1CFU each
100	850	1.23±0.17	691.05
250	2437	8.54±1.96	285.36
500	4657	20.34±2.48	228.95
1000	9600	30.58±3.71	313.93

isolated cells of range 15–45 × 10^4 from both the primary cultures. The cultures were routinely visualized under inverted phase contrast microscope. The cells started sprouting on 20–38 h itself in both *in vitro* cultures, grew more rapidly and reached 80–90% confluency on 7–8th day of culture.

Different techniques exist for isolation of MSCs from diverse sources. In this study, both a standard tissue digestion using trypsin and collagenase and MSC isolation by tissue explant techniques were performed. Enzymatic digestion by Trypsin and collagenase, first described by Rodbell (1964), is a widely used method for digestion and degradation of the collagen network of tissue. This method have a few disadvantages, such as, relatively high costs of enzymes, purity and purification of the enzymes, time-consuming labour and inconsistent results (Hefley *et al.* 1981, Williams *et al.* 1995, Hyder *et al.* 2005, Baptista *et al.* 2009). Several studies had reported the deleterious effects of enzymes on the phenotype and behaviour of cells (Liu *et al.* 2009, Patel *et al.* 2009). Therefore, in this current study, we considered a non-enzymatic isolation technique to recover MSCs, which would potentially be less affected and damaged than by an enzymatic isolation technique (Tsai *et al*. 2004, Sanchez-Guijo *et al*. 2009). Hence, the isolation of MSCs by explant technique was performed and compared to enzymatic digestion. The obvious benefit of this non-enzymatic cell isolation technique is that the procedure is simpler in comparison to the enzymatic method and does not require expensive enzymes. Only disadvantage with this method is that only cells located at the tissue border can migrate out of the tissue, so that not all MSCs residing in the tissue can be collected when using this technique. Another possibility to improve the MSC yield from tissue explant cultures might be to minimize the tissue size so that effective use of the explanted tissue can be done. In conclusion, in the current study, no significant differences were observed in terms of the growth patterns, colony forming units and morphology of the cells isolated from both the protocols. And this study represents the first step towards standardization of protocols for isolation and maintenance of MSCs in culture in order to create an equine stem cell bank which might help in regenerative medicine of horses.

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