ISOLATION AND CULTURE OF PUTATIVE MESENCHYMAL STEM CELLS FROM THE EQUINE AMNIOTIC MEMBRANE[#]

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ABSTRACT

Multipotent mesenchymal stromal cells (MSCs) are a promising therapeutic tool for the treatment of equine tendon and other musculoskeletal injuries. While bone marrow is considered the 'gold standard' source of these cells, various other tissues contain mesenchymal stem cells with potentially useful features. Equine foetal adnexa, amniotic membranes, chorion, wharton's jelly, umbilical cord blood (UCB) and amniotic fluid (AF) are potential non-invasive sources of MSCs. We collected amniotic membrane from twenty mares during their foaling for isolation of MSCs and evaluated them for the differences in isolation rates, proliferation capacity and expression of pluripotency markers. The plastic adherent colonies were observed in 80% of the collected samples. The proliferation potential of AM derived MSCs was measured based on their population doubling time (PDT). Amnion derived MSCs expressed CD29, CD44, CD73, CD90 and CD105 and were negative for haematopoietic and leukocytic markers (CD14, CD34 and CD45). These MSCs also expressed the *Oct-4*, *Sox-2* and *Nanog*, which are the pluripotency markers. The present study results indicated that amniotic membrane derived MSCs may be a good source of putative stem cells, making them potentially useful for veterinary regenerative medicine and cell-based therapy.

Key words: Amniotic membrane, equine, mesenchymal stem cells, pluripotency

Introduction

Mesenchymal stem cells (MSCs), also known as multipotent stem cells or mesenchymal stromal cells, have unique characteristics such as a fibroblast-like morphology, expression of specific surface markers and multipotent differentiation capacity (Díaz-Prado *et al*., 2011 and La Rocca *et al*., 2009). Human MSCs can be isolated from various tissues including bone marrow, adipose tissue, umbilical cord blood, Wharton's jelly, amniotic fluid and placenta (Kamishina *et al*., 2006).

In equines, several reports have demonstrated that isolation and characterization of stem cells could be possible using equine-specific tissues such as bone marrow, umbilical cord blood and adipose tissue (Arnhold *et al*., 2007, de Mattos *et al*., 2009). MSC are non-haematopoietic, multipotent progenitor cells that are easily isolated from various adult tissues. 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). Umbilical cord blood can serve as a source of stem cells without the need for painful harvesting methodologies. Nevertheless, studies of stem cells from umbilical cord blood are inadequate compared to ones evaluating stem cells from bone marrow and adipose tissue (De Schauwer *et al*., 2011). Similarly, amniotic membrane could also serve as a source of stem cells that are easily obtained on a large scale with minimal pain and suffering. The amniotic membrane, or amnion, is a layer of foetal membrane and one of the three placental layers (Parolini *et al*., 2008). During pregnancy, the placenta supports foetal development by supplying nutrients and oxygen to the uterine environment. Some reports have shown that the amniotic membrane can be used for healing wounds and corneal damage (Parolini *et al*., 2008). However, this membrane is usually treated as medical waste and discarded.

In the present study, we successfully isolated, cultured and characterized equine amniotic membrane-derived MSCs (AM-MSCs), and showed that these cells are capable of self-renewal and expresses the pluripotent markers. Additionally, the cells proliferated vigorously and displayed a similar morphology to that typical of human MSCs. Therefore, we infer that amniotic membrane may be a useful source of mesenchymal stem cells for veterinary cell-based therapy and regenerative medicine.

Materials and Methods

The present work has been 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 and until mentioned otherwise. All tissues were obtained *via* normal delivery without the use of any invasive methodologies. We used amniotic membranes that are normally discarded after delivery. The isolated membranes were only used for stem cell isolation and characterization.

Cell isolation and Culture

Cell isolation and culturing were performed as previously described with some modifications (Park *et al*., 2012). In brief, the collected amniotic membranes were washed with normal saline (0.9%) three to four times under sterile conditions to

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remove debris and blood. To detach and remove the epithelial layer from the amniotic membranes, the membranes were treated with 2% trypsin for 30 min at 37ÚC. After trypsinization, the membranes were washed with normal saline (0.9%) three times. After washing, the amniotic membrane was minced with a surgical blade and scissors. The minced tissue was digested at 37ÚC for approximately 2-3 h with collagenase type I (2 mg/mL). The digested samples were washed in phosphate-buffered saline and centrifuged at 350×g for 10 min. The cell pellet was resuspended in basal culture medium composed of low glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS). The cells were cultured in a humidified atmosphere with 5% CO_2 at 37.5ºC. The basal culture medium was changed two times a week and passaged after reaching 80% confluency.

Cryopreservability of cells and recovery

The MSCs obtained 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 37ÚC for 3 minutes 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 1000 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 and Culture media) and were initially stored on -80ºC and shifted to LN_2 storage cryocans on next day.

Cryovials were thawed in a 37°C water bath for 2 minutes 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 1000RPM for 4 minutes 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 in the viability and cell number of the MSCs isolated from the both the protocols.

Growth curve

Cells of the passage 6 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.

Population doubling time

The proliferation capacity of amnion derived MSCs were 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.

Charcterisation of the MSCs

The MSCs isolated from the both the protocols were characterized for the pluripotency gene expression (*Oct-4*) 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 $P<0.05$.Also statistical analysis 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

The amnion is the innermost of the two fetal membranes. It encloses the fetus, allowing it to move freely and providing protection from the external environment. The amniotic membrane has a thin, nonvascular structure with two layers: an epithelial monolayer and stromal layer (Yu *et al*., 2009). After parturition, the placenta is classified as medical waste and usually discarded. There are, however, clinical studies showing that the amniotic membrane can potentially be used to promote wound healing and corneal reconstruction (Kesting *et al*., 2008). Additionally, isolation and characterization of human MSCs from the amniotic membrane and whole placenta have also been reported (Parolini *et al*., 2008). These stem cells have tri-lineage differentiation and self-renewal capabilities, which are characteristic MSC features. MSCs have many advantages for use in cell-based therapy including immune privilege, an absence of associated ethical issues and no requirement of invasive procedures for harvesting the amnion. Recently, equine-derived stem cells were isolated and their potential use in cell-based therapy was examined (Frisbie *at al*., 2009; Schnabel *et al*., 2009). However, these investigations have been confined to limited stem cell sources such as equine adipose tissue, umbilical cord blood and bone marrow (Koch *et al*., 2007). Therefore, a greater diversity of stem cell sources is required. In the present study, we successfully isolated and characterized MSCs from AM. The collected amniotic membrane was enzymatically digested to recover the cells. After digestion, the AM-MSCs were seeded in basal culture medium until passage 14. Using this procedure, we obtained cells from four equine amniotic tissue samples. The rate of success was 100%, and all isolated cells (from all four samples) showed similar morphologies.

Morphological assessment

Cells isolated exhibited large and occasionally multi-nucleated cell types, we observed small, spindle-shaped and mostly mono-nucleated cell types in the primary culture. This heterogeneity cell population was no longer be observed at the second passage as the smaller spindle-shaped fibroblastoid cells started appearing to dominate and to 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 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 amniotic membrane had classic "S" shape involving a lag, log and plateau phases (Fig. 1). The population doubling time observed was approximately 40.2 h. 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 changes their morphology and the cells began to enter the plateau phase after the 8th day.

The AM-MSCs expressed CD44, CD90 and CD105. The MSCs isolated from the amnion, expressed the *Oct-4* in RT-PCR analysis and stained positive for the Alkaline Phosphatase staining (Data Not Shown).

The MSCs of larger animals (sheep, dog and horse) are usually used for a preclinical evaluation of joint tissue regeneration (Dragoo J. *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 foetal cells that could be easily used as multipotent stem cells. In this study, MSCs were successfully isolated from Equine amnion and expanded (Fig. 2). We isolated cells of range 15-45x 10^4 from both the primary cultures. The cultures were routinely visualized under inverted phase contrast microscope. The cells started sprouting on 20-38h itself *in vitro* cultures grew more rapidly and reached 80-90% confluency on 7-8th day of culture. In conclusion, in the current study there was not any significant differences were observed in terms of the growth patterns, colony forming units and morphology of the cells isolated. 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.

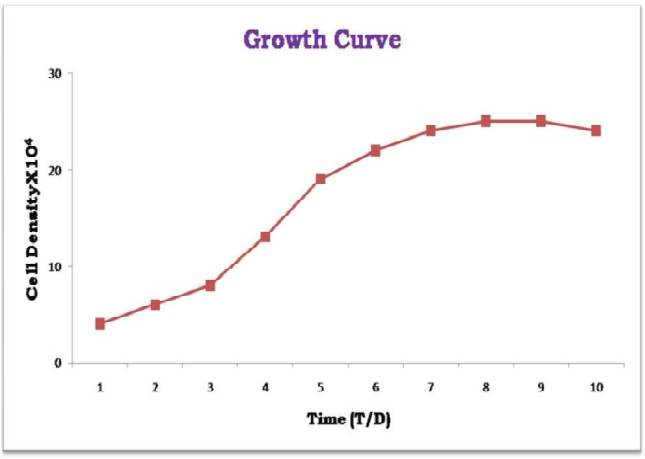

Fig.1: Growth curve of MSCs of Amniotic fluid (AF-MSCs)

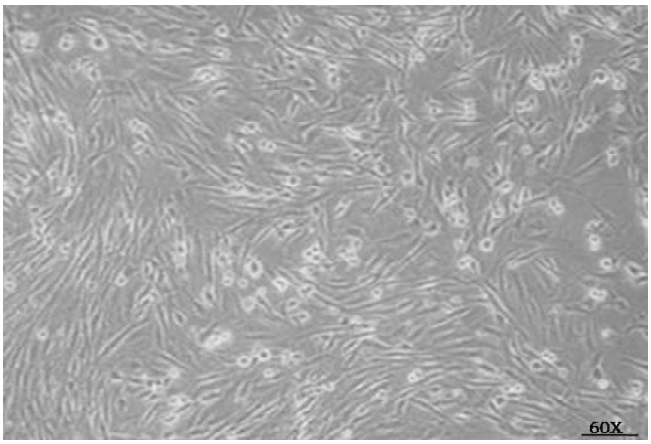

Fig. 2: Mesenchymal stem cells isolated from equine amniotic membrane

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