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CANINE AMNIOTIC MEMBRANE DERIVED MESENCHYMAL STEM CELLS- POTENTIAL SOURCES FOR REGENERATIVE MEDICINE

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Abstract

Canine mesenchymal stem cells (MSCs) can be defined with self renew potential and specific differentiation capacity. Amiotic membrane represent an important source of MSCs, which can be harvested by minimally invasive methods. The aim of our study was to evaluate the growth characteristics of canine amniotic membrane derived mesenchymal stem cells. The placenta samples were collected after cesarean section from healthy mixed breed dogs. MSCs isolation was performed using enzymatic method. Isolated cells were cultured in propagation medium: Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic-antimycotic (Sigma-Aldrich). The medium was changed after 4 days. The cell doubling number, cell proliferation capacity, cell doubling time, daily duplication rate and clonogenic efficacy were evaluated. Our study demonstrate the self renew potential of canine amniotic membrane derived mesenchymal stem cells, and can represent a potential source of stem cells for canine regenerative medicine.

Key words*: canine, stem cells, amniotic membrane, proliferation, cells growth.*

INTRODUCTION

Mesenchymal stem cells (MSCs), are multi-potent cells with unique immunoregulatory properties and self-renewal capacity (Ullah et al., 2015; Pall et al.; 2015; de Bakker E. et al., 2013). MSCs can be obtained from mesodermal tissues, endodermal tissues and ectoderm-derived tissues (Phinney et al., 2007; de Bakker E. et al., 2013).

According to the International Society for Cellular Therapy, MSCs are defined by their plastic adherence, expression of some specific markers and *in vitro* differentiation potential (Dominici et al., 2016).

For regenerative therapy in veterinary medicine, mesenchymal stromal cells (MSC) have been traditionally isolated from bone marrow or adipose tissue.

Neonatal tissues, normally are discarded after birth from all species (Saulnier et al., 2016; Seo et al., 2009).

These cells have been described as primitive cells with proliferative and immunosuppressive potential (Saulnier et al., 2016; Maymó et al., 2018).

The aim of our study was to evaluate the growth characteristics of canine amniotic membrane derived mesenchymal stem cells.

MATERIALS AND METHODS

Samples (n=3) were collected during cesarean-section deliveries from healthy mixed breed dogs. The samples were harvested after owner's agreement. The amniotic membrane was separated from chorionic membrane mechanically.

The samples were minced and treated with collagenase type I (Sigma-Aldrich) at 37℃ for 1 h. After centrifugation the cell pellet was resuspended in basal culture medium DMEM-F12 (Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Invitrogen, USA) 1% AA (antibiotic-antimycotic, Sigma-Aldrich).

The cells were incubated at 37°C in humidified atmosphere with 5% CO_2. After 4 days, non-adherent cells were removed and the medium was replaced.

The passages was performed at a confluence of 80-90%. The clonal capacity and population-doubling times (DTs) have been calculated.

The clonal capacity of canine amniotic tissue derived cells was assessed using CFU-F assay. $5x10^5$cells/well was cultured in basal medium, after 10 days the cells were fixed with 4% paraformaldehyde for 10 min and stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO) in 10% methanol for 20 minutes. Colony-

forming efficiency was calculated as percentage of the ratio of number of colonies counted to number of cells initially seeded. In order to evaluate the growth curves the cells were plated at a density of $4x10^3$ cells (six-well plate).
After every 72 h of culture, cells from one well were counted. proliferation rate was determined as previously reported (Lange-Consiglio et al., 2012; Corradetti et al., 2014).
The amniotic tissue derived cells (p1-p5) were plated at a density of $4x10^3$ cells/cm^2. Every 4 days the cells were trypsinized, counted and reseeded at the initial density ($4x10^3$ cells/cm^2).
Doubling time were calculated for each passage using the formula: doubling time (DT)=culture time/number of cell generations. Cell generations =log (Nc/No)/log2 (Nc, number of cells at confluence; No, the number of seeded cells).
For evaluation of cell phenotype, cell suspensions were incubated for 20 minutes at 4°C with CD34-FITC, CD45-FITC, CD90-FITC CD44-PE. Samples were analyzed with a FACSCanto II and Diva software (BD Biosciences).

RESULTS AND DISCUSSIONS

Collection of placental tissue during cesarean-section deliveries from canines did not have any adverse effect on the donors. Epitelial layer of amniotic tissue were separated and treated enzymatically. The cells suspension was cultured in standard propagation media. After 48h single cells with round, flatted, stellate, spindle shape morphology was observed. After 72 h the non adherent cells were removed.
Aderent fibroblast-like cells formed round clusters and after 14-15 days of culture, they reached 80 to 90 % confluency.
After several passages the degree of heterogeneity decreased, cells proliferated uniformly maintaining a homogeneous fibroblast-like morphology.
Total number of cells and cells viability was measured in each passages (Figure 1). In P1 the cells viability was 84.00±1.73 After the first passages the cells viability was 94.66±3.05.
The cell viability remained at approximately constant level, but after P3 cell viability decreased slightly.
The total number of cells also showed an increased tendency (Figure 1, 2).

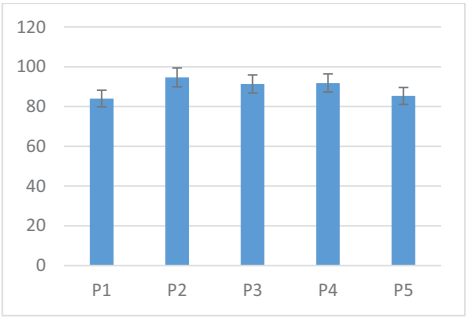

Figure 1. Canine amniotic tissue derived cells viability P1-P5

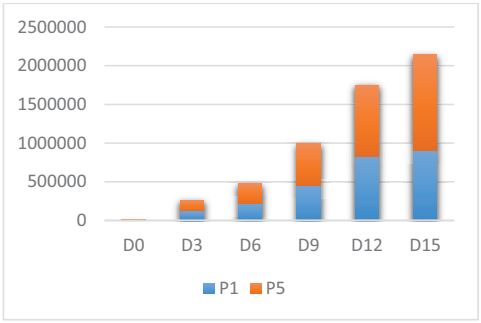

Figure 2. Total number of cells and cell viability P1-P5

The population doubling time (DT) at P1 was 1.26±0.11, 1.73±0.20 at P2 whereas at P5 the DT was 3.03±0.20 (Figure 3).

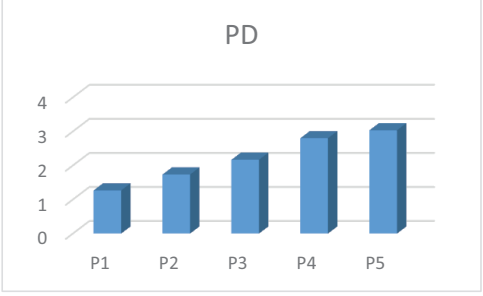

Figure 3. Doubling times at different passages (P1-P5)

The clonogenic potential of MSCs was assessed by CFU-F assay. Isolated cells displayed colony-forming ability; the frequency of colony forming cells was 42.88%.
The isolated cells expressed high levels of CD44 and CD90, and were negative for CD34, CD45 (Figure 4).

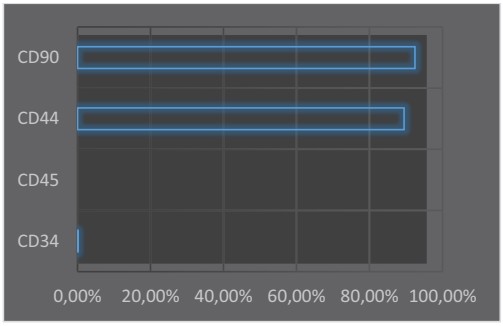

Figure 4. Flow cytometric analysis of canine amniotic tissue derived mesenchymal stem cells

The aim of our study was to identify a valuble sources of stem cells for regenerative medicine. Mesenchymal stem cells (MSCs) were first isolated from bone marrow (Noth et al., 2008). Recent study has identified alternative sources of MSCs, including umbilical cord blood (Martin-Rendon et al., 2008; Reed et al., 2008) adipose tissue (Eirin et al., 2012; Elashry et al., 2017), ovary (Trindade et al., 2017), placenta (Fukuchi, 2004; Fernandes et al., 2012; Yu et al., 2013), palatal tissue (Pall et al., 2017), dental pulp (Huang et al., 2009), synovial membrane (Hermida-Gomez et al., 2011), peripheral blood (Tondreau et al., 2005), periodontal ligament (Park et al., 2011), endometrium (Schwab et al., 2008), umbilical cord (Sarugaser et al., 2005), Wharton Jelly (Davies et al., 2017). The principal task of regenerative medicine is to identify a safe and valuable source of stem cells that can be used for treatment of different diseases (Si JW et al., 2015; Maymó et al., 2018). Stem cells derived from fetal tissues are an attractive source of cells for regenerative medicine (Noth et al., 2008; Parveen, 2018). Two types of stem cells can be isolated from the amniotic membrane: the stromal cells and the epithelial amniotic cells (Maymó et al., 2018). The presence of mesenchymal stem cells was described for the first time in human by Kaviani et al., 2001. Our study showed that mesenchymal stem cells could be successfully isolated form canine amniotic tissue.

CONCLUSIONS

Our results indicate a novel sources of stem cells with clonogenic and proliferative potential which can serve as a model system of mesenchymal stem cells for veterinary regenerative medicine.

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