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Spheroids from equine amnion mesenchymal stem cells: an *in vitro* study

Alessandra Coli[a], Maria Rita Stornelli[a], Francesca Nocchi[b], Roberta Lamanna[b], Mariacarla Iorio[b], Simone Lapi[b], Patrizia Urciuoli[b], Carla Lenzi[a], Elisabetta Giannessi[a]

Abstract

Background: Equine amnion mesenchymal stem cells (EAMSCs) from amnion isolated after the foal birth represented an alternative source of easy collection of mesenchymal cells used in equine regenerative medicine.

Materials and Methods: These cells grown as two-dimensional (2-D) culture in alpha modified minimum essential medium supplemented with epidermal growth factor were differentiated in adipogenic, chondrogenic, and osteogenic cells. Half a million cells as pellet were left in 15 ml tubes with the same differentiation media for 20 days. After the pellets were collected, embedded in paraffin for morphological study.

Results: 2-D culture showed EAMSCs with an embryonic phenotype (C-kit+, CD105+, Oct-4+) and a differentiation potential in adipogenic, chondrogenic and osteogenic multipotent cells. By a reproducible method of three-dimensional (3-D) culture, at day 20 the Authors evidenced a formation of small aggregated spheroids gradually gathering. In cross sections, the surface of the spheroid evidenced flattened cells embedded in a red matrix by Alizarin staining and occasionally a core of calcium precipitation. A network of apoptotic or necrotic cells in a not mineralized matrix was present into the center of nodules. The 3-D spheroids appeared larger (mean diameter of 605 ± 53 µm for gathering spheroids and 1486 ± 79 µm for spheroids already gathered) than those from standard monolayer cultures (mean diameter of 200 ± 73 µm).

Conclusions: EAMSCs cultured in 3D method preserve their *in vitro* multipotent differentiation than adherent 2-D culture method. These EAMSCs included in the extracellular matrix not mineralized at day 20 seem to be a good source of MSCs for tissue repair and regeneration in equine medicine.

Keywords: Equine, Amnion, Mesenchymal stem cells, Cell culture, Spheroid, Immunohistochemistry

INTRODUCTION

Equine mesenchymal stem cells (EMSCs) were isolated from bone marrow [1-4], adipose tissue [5], peripheral blood [1] and cord blood [6,7]. Currently, there is an increasing interest in the investigation of adult extra-embryonic tissues such as fetal adnexa (amnion, amniotic fluid and Wharton jelly) [8-10] due to easy collection after birth of the foal.

[a]Department of Veterinary Sciences, University of Pisa, Pisa, Italy, [b]Department of ???, Cell Biology and Tissue Regeneration Laboratory - Immunohaematology 2 Unit - Azienda Ospedaliero Universitaria Pisana, Pisa, Italy

Correspondence: Alessandra Coli, Department of Veterinary Sciences, University of Pisa, Viale delle Piagge, 2, 56124, Pisa, Italy. Tel.: +390502216856, webfax: +390502210655, Email: alessandra.coli@unipi.it

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Hoyonowski et al. [11] first isolated and characterized mesenchymal stem cells from equine umbilical cord matrix (Wharton's jelly) with an embryonic phenotype (Oct-4, C-kit) and an ability of osteogenic, chondrogenic and adipogenic differentiation. Among the equine fetal adnexa, the amnion has been recently studied as an alternative source of mesenchymal cells used in the field of equine regenerative medicine. The amnion is a thin membrane, which forms the wall of a fluid-filled sac in which the embryo develops. In mammals, the amnion is derived from the inner somatopleure membrane, which remains attached to the embryo at the umbilicus. In literature, it is reported that mesenchymal cells can be isolated from amnion. They express stem cell surface markers such as embryonic stem cells and are doubly negative for MHC I and MHC II [12]. They can differentiate into ectodermal and endodermal lineages once isolated and grown in special culture media [13]. These cell populations display a fibroblast-like appearance, adhere onto plastic culture vessels, form clonal colonies and under appropriate culture conditions, they differentiate into adipocytes, osteocytes, chondrocytes and neuronal cells [14-17]. Therefore, these cells represent a potential use in cellular therapy and regenerative medicine applications. The Authors

have already performed a procedure of expanding equine amnion mesenchymal stem cells (EAMSCs) by a non-invasive technique for the isolation of the cells and used culture media supplemented with epidermal growth factor (EGF) [15,16].

In addition to traditional two-dimensional (2-D) culture methods in which the mesenchymal stem cells grow in a single layer, in recent years many authors suggest the use of three-dimensional (3-D) culture. As the plastic surface limits the potential of stem cells to recreate *in-vivo* conditions [18], aggregates of cells grown in suspension culture and showing a spheroid shape are reported. They can be embedded in scaffolds [19-22] or cultivated scaffold-free [23,24] though it is reported that a synthetic material may represent a limitation to the cell-cell interactions [25,26].

These spheroids ("mesenspheres") show a physiological microenvironment in which cell-cell or cell-scaffold interactions are better achieved than in monolayer culture methods [23,27]. If grown in appropriated culture media, these spheroids exhibit chondrogenic, adipogenic and osteogenic competence and an enhanced secretion of tropic factors [28-30].

Objective

The aim of this study is to perform an efficient and reproducible method for the isolation of EAMSCs and their differentiation into spheroids, reporting on adipogenic, chondrogenic and osteogenic differentiation in terms of morphological and morphometrical point of view, in order to obtain a biological source with potential clinical use in regenerative medicine of horses.

MATERIALS AND METHODS

Amnion cells were obtained from 4 10 to 13 years old standardbred mares as previously described [16]. For the study of cellular stemness, immunocytochemistry on amnion sections with anti-C-Kit, -CD105 and -Oct-4 antibodies, involved self-renewal of embryonic stem cells, was performed as reported above [16]. Amnion samples, washed in phosphate-buffered saline (PBS) solution (Euroclone, MI, Italy) and soaked in 10 mL of a collagenase solution (1 mg/mL) for 30 min at 37°C, were suspended and filtered through a 100 mm filter (Millipore, Billerica, MA, USA).

2-D Cell Culture

Nucleated cells isolated by gradient centrifugation (500 g for 10 min) were resuspended in Alpha modified minimum essential medium (α-MEM) (Cambrex, NJ, USA) with 10% fetal bovine serum (Eurobio, France), 10% horse serum (HS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (Euroclone, MI, Italy) and 10 ng/ml EGF (Sigma). Cells were cultured at 10^5 cells/cm^2 and the adherent ones were grown in fresh medium for about 14 days, until 90% of confluence (Passage 0, or P0). For further expansion (Passage 1, or P1) the cells were then sub-cultured (by 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid [EDTA]) (Euroclone, Milan, Italy) for 5 min at 37°C, replated at 5000 cells/cm^2 and harvested with the same protocol.

3-D Cell Culture

Half a million cells were dissociated with 0.25% trypsin in 1 mM EDTA (Euroclone, Milan, Italy), centrifuged at 1000 rpm for 5 min and the pellet was left in 15 ml tubes in a rotary shaker in an incubator with the differentiation media (adipogenic, chondrogenic and osteogenic media) for 20 days. Every 3 days, the tubes with the cells were centrifuged at 1000 rpm for 5 min, the supernatant removed and replaced with fresh medium. After 20 days, the pellets were collected and washed in PBS.

Flow Cytometric Study

Analysis was performed, as mentioned above, [16] using anti-MHC I (Serotec, Oxford, UK), anti-MHC II (Serotec), anti-CD14 (Serotec), anti-CD45 (Serotec), anti-CD44 (Chemicon, Temecula, CA, U.S.A.), anti-b-1-integrin (Chemicon) and anti-CD90 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) mouse monoclonal antibodies. As secondary antibody, an FITC goat anti-mouse IgG (Santa Cruz Biotechnology) was used. A minimum of 10.000 events was acquired for each sample.

Differentiation protocols

Adipogenic medium: a-MEM supplemented with 10% FCS and 10% HS, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 12 mM L-glutamine, 5µg/mL insulin (Lilly), 50µM indomethacin (Sigma), 1 mM dexamethasone (Sigma, St.Louis, MO, U.S.A.) and 0.5 µM 3-isobutyl-1-methylxanthine (IBMX, Sigma, St.Louis, MO, U.S.A.) for 2 weeks.

Chondrogenic medium: Chondrocyte basal medium (Cambrex Bio Science, Walkersville, MD, U.S.A.) for 3 weeks.

Osteogenic medium: a-MEM supplemented with 10% FCS and 10% HS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 20 mM b-glycerol phosphate (Sigma, St.Louis, MO, U.S.A), 100 nM dexamethasone (Sigma, St.Louis, MO, U.S.A.) and 250µM ascorbate 2-phosphate (Sigma, St.Louis, MO, U.S.A) for 3 weeks.

Histology

Cell monolayer (2-D cell culture) was fixed in 10% formalin for 20 min at RT and stained with 0.5% Oil Red O (Sigma, St. Louis, MO, U.S.A.) in methanol (Sigma) for 20 min at RT, Alcian Blue solution (Sigma, St. Louis, MO, U.S.A.) pH 2.5 for 20 min at RT (cell nuclei counterstained with Weigert's iron hematoxylin) and Alizarin Red (Sigma, St. Louis, MO, U.S.A.) pH 4.1 for 20 min at RT.

The spheroids from 3-D culture were fixed in 10% formalin for 20 min at reverse transcription (RT) and embedded in

paraffin. They were sectioned at 4 μ, made permeable with methanol for 2 min at RT and washed 3 times with PBS. The sections were processed by Alizarin Red and by double staining (Alcian blue and Alizarin Red).

Morphometry

The size of spheroids was measured by Nikon Digital Sight DS-U1 Program (Nikon)

RESULTS

Amnion sections showed positivity for C-Kit, CD105 and Oct-4 antigens, markers of stemness (Figure 1). Cytofluorimetric analysis of EAMSCs showed cells positive to CD 90, CD 44, CD 14 and CD 45.

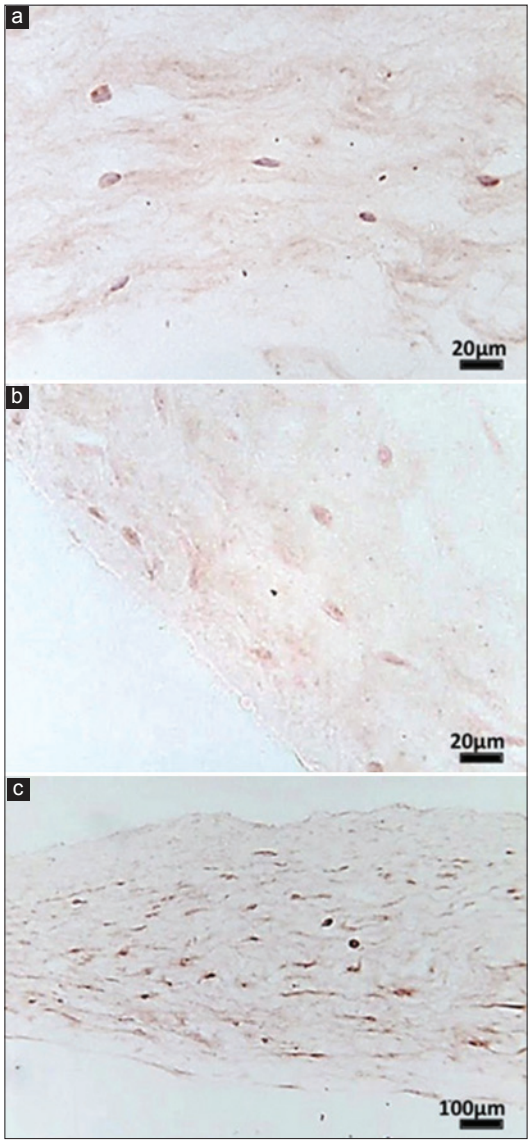

Figure 1: Immunohistochemical study: C-Kit (a), CD105 (b) and Oct-4 (c) antigens

In 2-D cell culture adipogenic differentiation showed rounded cells staining positive with Oil Red O, with lipid vesicles in the cytoplasm increasing in amount over time (Figure 2a). Chondrogenic differentiation showed glycosaminoglycans in the matrix after alcian blue staining (Figure 2b). With osteogenic differentiation, from the 5th day of cell culture, the Authors observed bone nodules (200 ± 73 μm of mean diameter) with a layer of epithelium-like fibroblastoid cells around, close to each other, more elongated and flattened (Figure 2c and d). AQ6

Histological analyses of 3-D cell culture (Figure 3) after 20 days of culture showed nodules aggregated in small spheroids that gradually gathered into a single one (Figure 3a). Epithelial cells of spheroid surface, embedded in a red matrix by Alizarin staining, were observed. In every single small nodule it was possible to observe epithelium-like fibroblastoid cells and a network of apoptotic or necrotic cells embedded into a

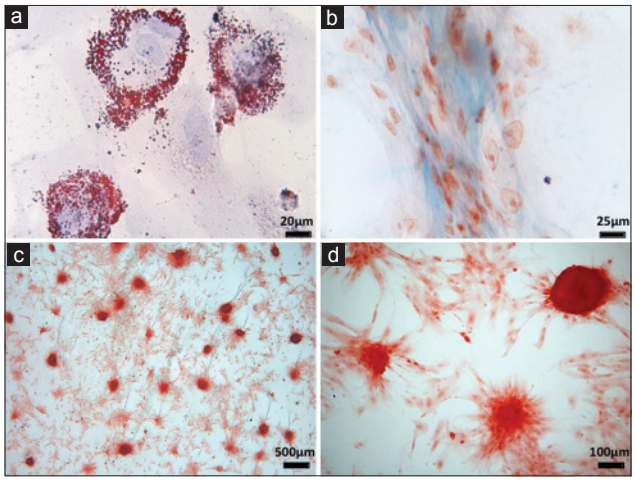

Figure 2: EAMSCs in 2-D culture: adipogenic (oil red O) (a), chondrogenic (alcian blue) (b) and osteogenic (alizarin red) (c and d) differentiation

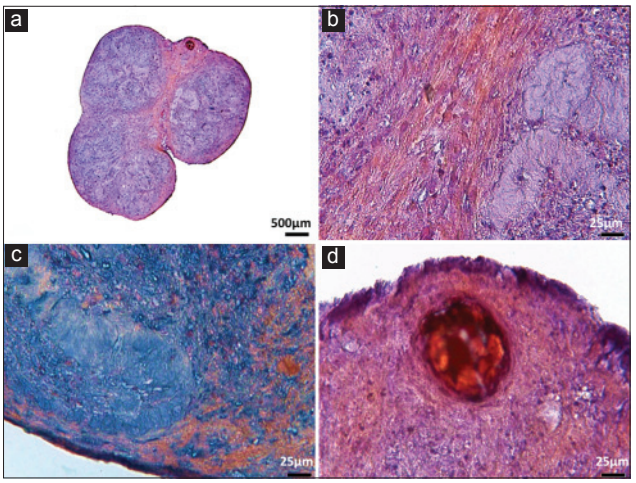

Figure 3: Equine amnion mesenchymal stem cells differentiation in 3-D culture spheroid sections: Alizarin red (a, b and d); alcian blue and alizarin red (c)

non-mineralized matrix, organized in circular or ovoid areas, positive to Alcian blue staining, (Figure 3b and c). Where the matrix was mineralized, it was possible to highlight a core of calcium precipitation (Figure 3d). The 3-D spheroids appeared larger than those from standard monolayer cultures: Their mean diameter ranged from 605 ± 53 μm for single spheroids getting together to 1486 ± 79 μm for spheroids already gathered in a single one.

DISCUSSION

In the literature are present studies about equine mesenchymal cells from other sources but not from amnion [6,10]. The EAMSCs were investigated for their possible use in cell therapy because of non-invasive techniques for cell extraction; indeed, it was easy to sample amnion immediately after birth because the colt come off the chorion and the mare second the rest of the placenta later. Bacterial and fungal contamination represented the main problem to be settled, due to the environment where the procedure of amnion sampling was performed. For this purpose we used a sampling protocol employed in our previous study [15] Cytofluorimetric analysis showed a phenotype of mesenchymal stem cells, confirming the positivity of stemness antigens and thus their pluripotency.

When grown in specific culture media, EAMSCs differentiated into adipogenic, chondrogenic and osteogenic lineages. The effects of the addition of EGF in the culture medium did not affect their ability to differentiate [15]. This study developed a technique for sampling, isolation and expansion of EAMSCs for producing a 3-D cell culture system. The method of aggregation for EAMSCs spheroid formation yielded largely homogeneous spheroids, which might be maintained for a longer time (20 days) in suspension by rotary shaking. Under chondrogenic conditions the Authors showed the presence of glycosaminoglycans as blue deposits immersed in a network of fibroblastoid cells, as reported in literature for bovine umbilical cord [24] but of larger diameter for they gathered in a single spheroid. Under osteogenic conditions a significant positive staining of flattened cells suggesting their osteogenic differentiation was observed, even if the mineralization of nodule matrix was not complete at 20 days (as reported by double staining). By both of the staining methods, the Authors observed changes in cell morphology, especially for the cells embedded in the matrix, referred to as different replicative ability [18]. From literature, 3-D cell culture systems allowed a more physiological environment for stem cells survival in contrast to adherent monolayer; cell-cell and cell-matrix interactions improved. The matrix mineralization increased until day 14 of osteogenic differentiation for murine bone marrow-derived MSCs [23] and until day 10 for human bone marrow-derived MSCs [22]

CONCLUSIONS

Our data showed that at 20 days of osteogenic differentiation matrix mineralization is not yet complete (extracellular matrix was positive to alcian staining and negative to alizarin red staining). The Authors referred this difference to the embryonic origin of these cells; their self-renewal and high potential of sub-culturing *in vitro* might be greater than adult MSCs. This study confirmed the successful development of 3-D culture method for EAMSCs in preserving their *in-vitro* multi-potent differentiation than the adherent 2-D culture method. These results might provide a basis for the establishment of a database for collecting and preserving EAMSCs to be used for equine regenerative medicine.

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AQ2: Kindly provide department
AQ3: Kindly provide history details
AQ4: Embryonic stem cells are surface markers?
AQ5: I dont get this
AQ6: Meaning unclear