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Ultrastructural morphology of equine adipose-derived mesenchymal stem cells

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Summary. Mesenchymal stem cells are a virtually ubiquitous population of adult stem cells, able to differentiate into various tissue lineages. As they are multipotent and easy to grow in culture, they are at present considered very attractive candidates for tissue repair and gene therapy. With the exception of a few reports, mesenchymal stem cell morphology has been widely disregarded in the past years. In this paper we discuss the establishment of mesenchymal stem cell cultures from equine adipose tissue and describe their fine structure by transmission electron microscopy. The cultured cells revealed a fibroblastoid appearance and were characterized by an eccentric nucleus with multiple nucleoli, dense cytoplasm rich in ribosomes, a rough endoplasmic reticulum with dilated cisternae, elongated mitochondria and heterogeneous vacuolar inclusions. In addition, they were often interconnected by adhesion structures located on the cell body and on cytoplasmic processes contacting other cells. The features observed are evocative of an undifferentiated cellular phenotype and of an intense synthetic and metabolic activity.

Key words: Horse, Mesenchymal stem cells, Adipose tissue, Ultrastructure

Introduction

Mesenchymal stem cells (MSCs), a well-known type of adult stem cells, have generated great excitement and promise in the last thirty years as potential protagonists of cell-based therapies. Friedenstein and colleagues first described this population of bone marrow stromal cells that were able to adhere to the plastic culture substrate, giving rise to colonies of fibroblast-like proliferating elements (Friedenstein et al., 1974, 1976). Mesenchymal stem cells were subsequently isolated from different tissue sources such as periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle, spleen, lung, skin, blood vessels and umbilical cord blood, demonstrating their ubiquitous distribution *in vivo* (Van den Heuvel et al., 1987; Erices et al., 2000; Zvaifler et al., 2000; Zuk et al., 2001, 2002; Covas et al., 2003; Meirelles et al., 2006; Crigler et al., 2007; Yoshimura et al., 2007). Whatever their site of origin may be, MSCs are defined by several key characteristics: they strongly adhere to a plastic surface in culture; they are clonogenic; they show a surprising proliferation rate (self-renewing); finally, they exhibit a remarkable plasticity, being able to differentiate into many mature cell types of mesodermal and non mesodermal origin (multipotency) (Bruder et al., 1997; Pittenger et al., 1999; Woodbury et al., 2000; Payushina et al., 2006; Banas et al., 2007). Moreover, due to their low immunogenicity and lack of alloreactivity they are viewed as being optimal candidates for transplantation procedures (Bartholomew et al., 2002; Gotherstrom, 2007; Nauta and Fibbe, 2007).

Numerous reports have undertaken the study of the MSC immunophenotypic profile. The most frequently cited surface markers for MSCs are Stro-1, CD13, CD29, CD44, CD73, CD90, CD105 and CD106 (Gronthos et al., 2001; Dominici et al., 2006; Schäffler and Büchler 2007; de Mattos Carvalho et al., 2009).

However, in contrast with stem cells from different sources (e.g. haematopoietic stem cells), the immunophenotype of MSCs is far from being completely clarified, and discrepancies among different species, tissue sources or methods of analysis do emerge on evaluation of the literature. (de Mattos Carvalho et al., 2009; Mambelli et al., 2009).

Great attention has recently been paid to the study of

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MSCs in domestic animals: an increasing number of reports deal with the isolation and characterization of MSCs in several species, including horses (Fortier et al., 1998; Arnhold et al., 2007; Behr et al., 2007; Lee et al, 2007; Vidal et al., 2007; Giovannini et al., 2008; Colleoni et al., 2009; de Mattos Carvalho et al., 2009; Khatri et al., 2009; Zucconi et al., 2009). Much of the interest in this last species is focused on the use of MSCs for the treatment of orthopaedic injuries: bone marrow-derived MSCs have been experimentally used for the therapy of bone defects (Carstanjen et al., 2006) and cartilage repair (Wilke et al., 2007). However, by far their most frequent use has been in the treatment of tendon injuries (Herthel, 2000; Smith et al., 2003; Richardson et al., 2007).

More recently, adipose-derived MSCs (AdMSCs) have been proposed as a feasible alternative to bone marrow ones in such clinical applications (Vidal et al., 2007). Adipose tissue, in fact, allows extraction of a large volume of tissue without requiring any potentially painful procedure. Moreover, in humans, it is populated by a significantly higher number of multipotent stromal cells (1 out of 100 cells of the stromal fraction versus 1 out of 50-100 000 of whole bone marrow) (Fraser et al., 2006) that can be extensively and stably propagated in vitro in an undifferentiated state. Then, given the right signals, AdMSCs are able to undergo at least adipogenic, osteogenic and chondrogenic differentiation (Vidal et al., 2007, 2008).

Given the medical implications in human and veterinary medicine and considering the fervid and often controversial scientific debate regarding the nature of these cells and their post-transplantation fate, we hereby intended to provide a detailed morphological description of cultured horse AdMSCs observed by transmission electron microscopy (TEM). In fact, while most studies have focused on isolation, *in vitro* characterization and immunophenotyping of MSCs, their fine morphology has been documented only in a few reports regarding bone marrow, amnion and chorion term placenta, human adult blood vessels (Colter et al., 2001; Raimondo et al., 2006; Pasquinelli et al., 2007a,b; Li et al., 2008), but not on adipose tissue as cell source. Indeed, we believe that a thorough understanding of AdMSCs' fine structure - as well as the study of their functional characteristics - is an essential step in defining the features and the phenotypic stability of cells to be employed in transplantation procedures.

Materials and methods

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, Mo) unless otherwise specified. Type I collagenase was obtained from Worthington Biochemical (Lakewood, NJ). The fetal bovine serum (FBS) and the Dulbecco's modified Eagle's medium (DMEM) were from Gibco (Gaithersburg, Md). Glutaraldehyde was from Fluka (St. Louis, Mo, USA) and epossidic resin for sample inclusion was from Electron Microscopy Sciences (Hatfield, Pa). CD90 antibody was purchased from VMRD Inc. (Pullman, Wa), while the secondary antibody labelled with Texas Red was from Santa Cruz Biotechnology (Santa Cruz, Ca).

Cell isolation and culture

Equine adipose tissue samples were obtained from the subcutaneous fat of 2 donors and collected during surgery under general anaesthesia. Both subjects were 1 year old. The samples were transported to the laboratory in phosphate-buffered saline (PBS) within 3 hours post-surgery and processed according to previously published methods. Briefly, tissue samples were washed extensively with sterile PBS with the addition of 100 U/ml penicillin, 100 μg/ml streptomycin, 12.5 μg/ml amphotericin B to remove debris and red blood cells. The tissue was then finely minced and digested with 0.075% collagenase type I in a shaking water bath at 37°C for 30 minutes. Enzyme activity was neutralized by adding Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (complete basal medium) and centrifuged at 600g for 7 minutes to obtain a pellet. The supernatant containing adipocytes was discarded, while the precipitated stromal vascular cell fraction (SVCF) was resuspended in 0.83% NH_4Cl and incubated for 10 minutes at room temperature to lyse red blood cells. The SVCF was then collected by centrifugation and seeded in tissue culture flasks in complete basal medium. The cells were incubated at 37°C with 5% CO_2 (passage 0). Following 72h of incubation, the cells were washed with PBS and maintained in basal medium until they achieved 80% confluence. At this time, they were harvested using 0.5 mM EDTA/ 0.05% trypsin and passaged at a density of 5-10 000 cells/cm^2 (Passage 1). The cells were maintained in complete basal medium until passage 3. Then, to verify that the selected population comprised mesenchymal multipotent stem cells, CD90 reactivity was tested and a set of differentiation experiments was performed.

Finally, cells were prepared for transmission electron microscopic observation.

Adipogenic differentiation assay

At passage 3, the cells were harvested using trypsin/EDTA and plated with complete basal medium at a density of 30,000 cells/cm^2. They were allowed to expand until confluence before switching to adipogenic medium (day 1). This consisted of DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), 10 μg/ml insulin, 100 μM indomethacin (induction medium – IM). The cells were

fed with IM for 6 days, changing the medium every 2 days. At day 7, IM was replaced with a maintaining medium (MM) composed of DMEM, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 μg/ml insulin. This 8 day cycle of treatment was repeated 3 times. Then, the cells were fixed in 10% formaldehyde solution for 10 minutes, rinsed in PBS and stained with Oil Red 0.

Osteogenic differentiation assay

At passage 3, the cells were harvested using trypsin/EDTA and plated with complete basal medium at a density of 30,000 cells/cm_2. After attachment to the substrate, they were allowed to expand until confluence. To induce osteogenesis (day 1) the cells were fed with DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 μM dexamethasone, 10 mM glycerophosphate and 0.2 mM ascorbic acid 2-phosphate. The cultures were fed every three days throughout the study. After 18 days, they were fixed in 10% formaldehyde solution for 10 minutes, rinsed in PBS and stained with Alizarin Red.

Chondrogenic differentiation assay

Chondrogenesis was induced in micromass pellet cultures prepared with $2x10^6$ cells placed in a 15 ml polypropylene conical tube and centrifuged at 600g for 7 minutes. The pellet was cultured at 37°C with 5% CO_2 in 2 ml of chondrogenic medium, consisting of complete basal medium supplemented with 10 ng/ml of TGF-ß1, 0.05 mM ascorbic acid-2-phosphate and 6.25 μg/ml insulin. The medium was replaced every 2-3 days. Following a 4 week incubation period, the pellet was fixed in 10% neutral buffered formalin for 3 hours at room temperature, dehydrated through graded ethanol to xylene and embedded with paraffin. Five μm sections were prepared and stained with alcian blue.

Immunofluorescence

Subconfluent monolayers of cells at passage 3, grown on coverslips, were fixed with 4% paraformaldehyde for 20 minutes at 4°C. After several washes with PBS, the cells were treated with 10% goat normal serum in PBS for 30 min at room temperature (RT) to suppress non specific binding of the immunoglobulin and then incubated overnight at RT with anti-CD90 primary antibody diluted 1:100 in PBS. The appropriate Texas Red-conjugated secondary antibody, diluted 1:200 in PBS, was applied for 1h at RT.

Cells treated with the omission of the primary antibody were used as negative control. The cells were finally washed with PBS, mounted with a water-based mounting medium and then observed under an epifluorescent microscope (Leica, Germany).

Electron microscopy

AdMSCs cultures were daily observed under an inverted light microscope (Olympus CK2). Ultrastructural investigation was carried out at P3 (about 21-24 days of culture); at this time, the medium was discarded from the wells. Monolayer cultures were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.3, for 2 h at room temperature, detached from the well by means of a cell scraper and centrifuged at 600g for 10 minutes to remove the fixative. The pellet was subsequently washed twice in phosphate buffered saline (PBS), post-fixed in 2% osmium tetroxide, dehydrated in a graded series of ethanol up to absolute, preinfiltrated and embedded in Epon 812.

Ultrathin sections (90 nm) were mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined by means of a Philips EM 208

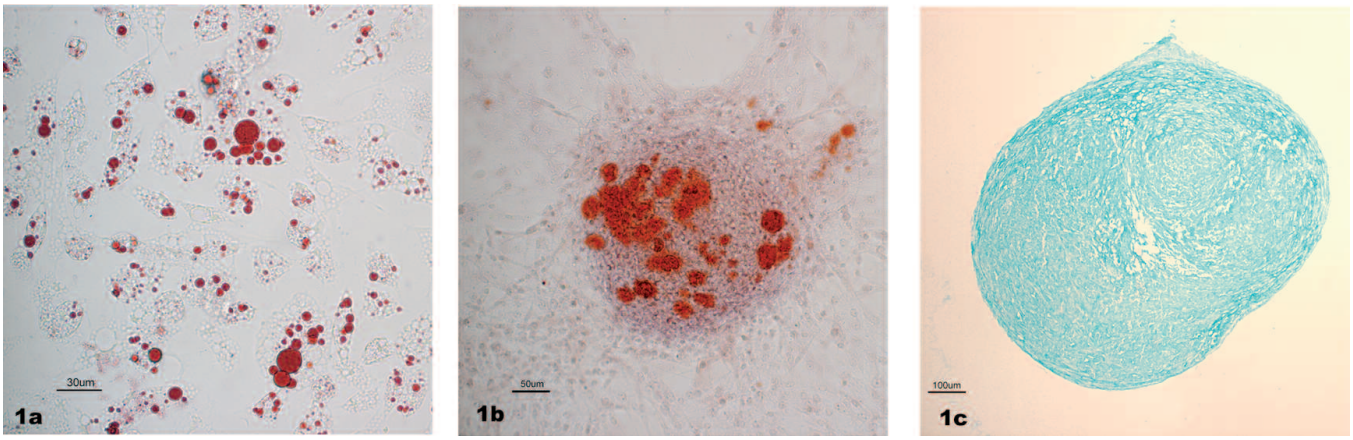


Fig. 1. A. Adipogenic differentiation assay. Notice the accumulation of lipid droplets within the cytoplasm of Ad-MSCs stained with Oil red O dye. **B.** Osteogenic differentiation was confirmed by the presence of areas of mineralization stained by Alizarin Red. **C.** Chondrogenic differentiation was demonstrated by Alcian Blue staining of paraffin-embedded pellet cultures.

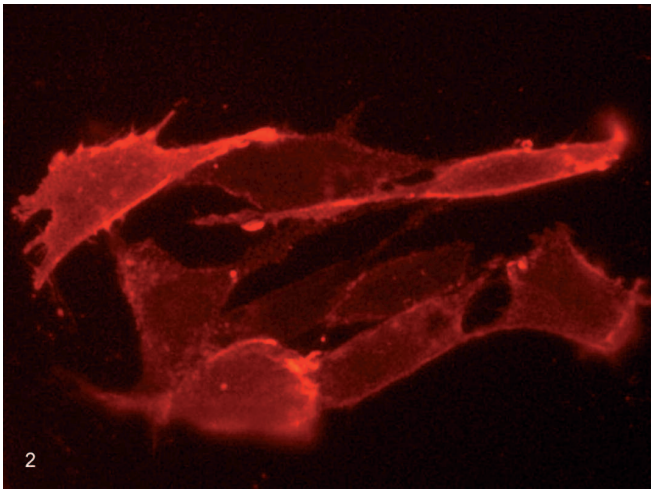
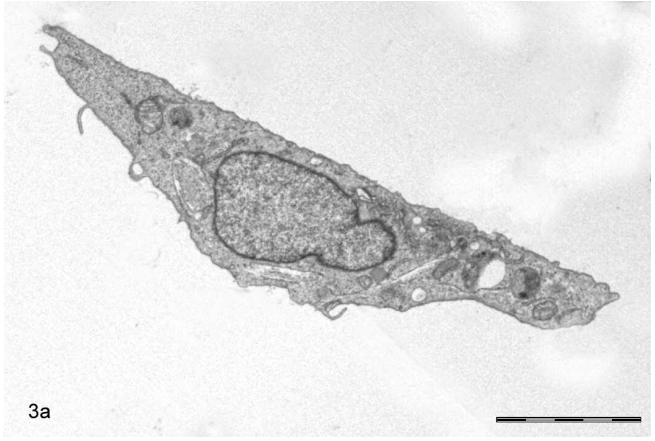


Fig. 2. Reactivity of equine Ad-MSCs to anti-CD90. Indirect immunofluorescence.



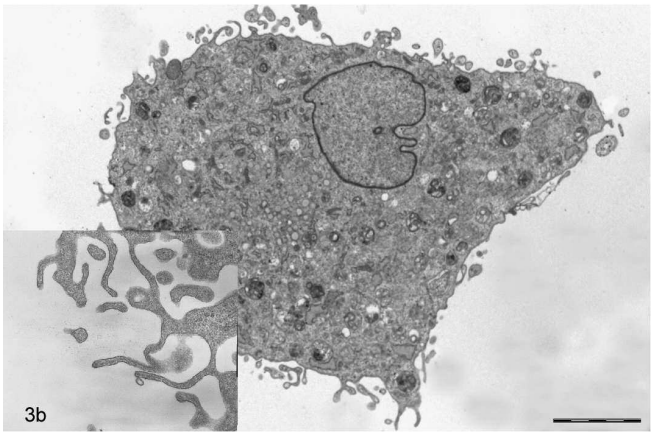


Fig. 3. Adipose-derived mesenchymal stem cells exhibiting the typical spindle **(a)** or irregularly polygonal **(b)** shape. In the insert, a detail of the cellular membrane characterized by dendritic filopodia. Scale bar: 5 μm.

transmission electron microscope equipped with a digital camera. (Center for Electron Microscopy (CUME), University of Perugia).

Results

Differentiation assays

When treated with appropriate adipogenic induction factors, a conspicuous fraction of equine AdMSCs accumulated intracellular lipids as multiple droplets; these could be stained for neutral lipid with Oil Red O dye (Fig. 1a). The lipid accumulation indicated that the cells had undergone adipogenic differentiation. No lipid droplets were observed in untreated negative controls.

Osteogenic differentiation of Ad-MSCs was characterized by the formation of multilayered, densely packed cellular aggregates. Mineralization was confirmed by the use of Alizarin Red stain. Calcium deposits appeared as red areas within the cellular nodules (Fig. 1b). In contrast, no calcification was observed in untreated control cells.

Chondrogenic differentiation was induced *in vitro* using a micromass culture technique. Equine Ad-MSCs, treated with inducing agents, formed a nodular cell aggregate comprising an extracellular matrix rich in sulphated proteoglycans that were specifically evidenced using Alcian blue staining (Fig. 1c).

Immunofluorescence

As previously demonstrated (Pascucci et al., 2007) using an indirect immunofluorescence procedure, the majority of the cells were positively stained for CD90, a reliable surface marker of MSCs (Fig. 2).

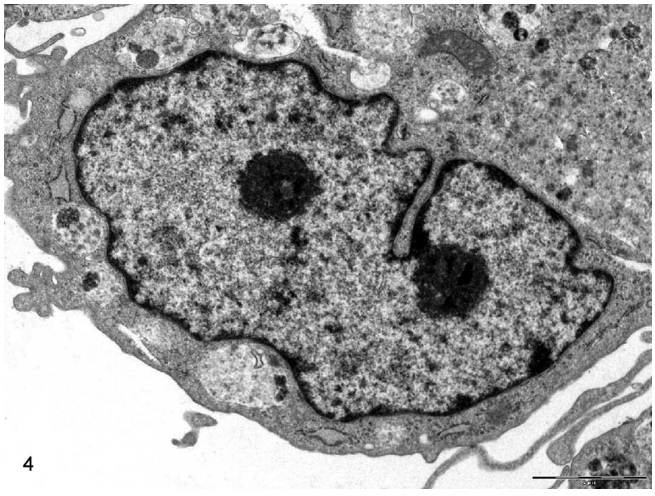


Fig. 4. A large, pale and eccentric nucleus characterized by variable deep clefts of the nuclear envelope and by a typical euchromatic aspect. Note the two prominent nucleoli. Scale bar: 2 μm.

Ultrastructural findings

When fed with DMEM supplemented with 10% FBS, equine AdMSCs easily expanded *in vitro* exhibiting a typical fibroblastoid shape. The cells were maintained in standard culture conditions for 21-24 days (3rd passage), so that a homogeneous cell population containing the putative stem cell fraction could be obtained.

On transmission electron microscopy, the cells were characterized by an elongated or irregularly polygonal shape (Fig. 3a,b).

They exhibited a large, pale, eccentric and euchromatic nucleus with a deeply indented outline. Nucleoli were usually multiple (2 or more) and prominent, sometimes located close to the nuclear envelope (Fig. 4).

The cellular membrane showed an irregular surface due to the presence of pleomorphic cytoplasmic protrusions such as pseudopodia and filopodia. Filopodia, in particular, appeared to be slender and dendritic and they were sometimes connected to adjacent cells (Fig. 3b, insert).

The cytoplasm exhibited a granular appearance due to the widely spread abundance of free ribosomes and polyribosomes forming concentric and spiraloid chains.

Cellular organelles were distributed throughout the cytoplasm. The rough endoplasmic reticulum (RER) was prominent and often dilated to give rise to large cisternae containing moderately electron-dense material (Fig. 5).

Mitochondria were numerous and homogeneously distributed throughout the cytoplasm; they were occasionally organized in small groups of 4-5 elements. They mostly appeared elongated and showed a dense matrix. The Golgi apparatus was well developed: it was comprised of flattened cisternae, transport and transition vesicles, as well as heterogeneous sized vacuoles, some of which were very large, containing low electron-dense amorphous material and originating from the trans Golgi region (Fig. 6).

The cytoplasmic compartment was populated by a noticeable amount of endosomal elements, such as

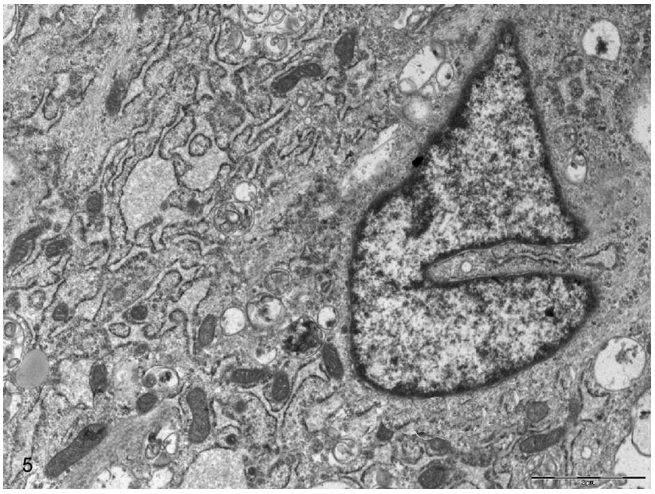


Fig. 5. The figure shows the well developed rough endoplasmic reticulum. It is characterized by bridge-connected stacks of cisternae; some of them exhibit a noticeable swelling and contain a moderately electron-dense material. Scale bar: 2 μm.

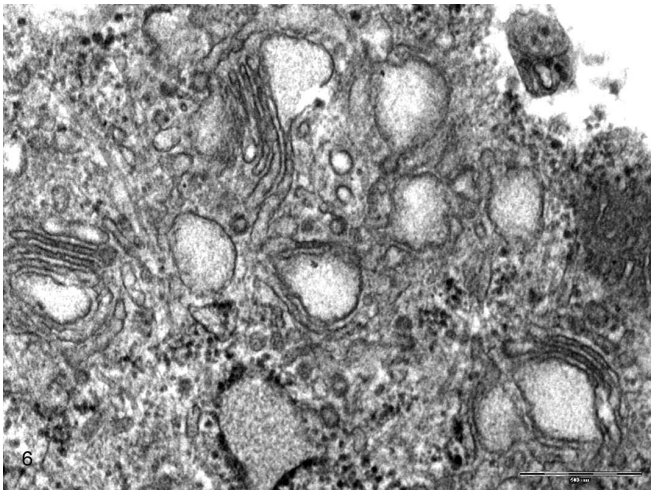


Fig. 6. The Golgi complex is made up of a stack of flattened membranous sacs, transport and transition vesicles as well as heterogeneous sized vacuoles. The last ones are sometimes very large and contain low electron-dense amorphous material. Scale bar: 500 nm.

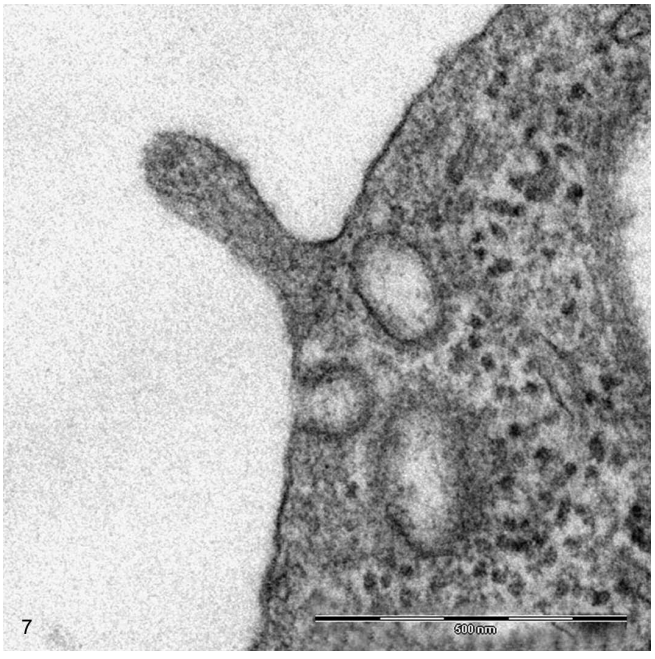


Fig. 7. Coated pits and endocytic vesicles located beneath the plasma membrane. Scale bar: 500 nm.

primary endocytic vesicles, early endosomes, late endosomes, endolysosomes and lysosomes. Endocytic vesicles showed an average diameter of 100-150 nm and were essentially located beneath the plasma membrane, being mainly generated at the cell surface as coated pits (Fig. 7). Early endosomes appeared as single compartment membrane-bound vesicles which frequently fused with endocytotic vesicles (Fig. 8).

Late endosomes, also referred to as multivesicular endosomes or multivesicular bodies (MVBs), showed a multiple-compartment structure and appeared as large vacuoles (500 nm and more) containing 30 to 70 nm wide intralumenal small vesicles (Fig. 9). The number of these vesicles increased on the gradual maturation of late endosomes (Fig. 10). Endolysosomes, derived from the fusion of endosomes with Golgian hydrolasic vesicles, were also observed; they appeared as large vacuolar inclusions characterized by a heterogeneous content: vesicles, typical of late endosomes, were mixed with aggregates of electron-dense material (Fig. 11). Early autophagosomes were also seen; they exhibited a double membrane embracing small areas of organelles containing cytoplasm (Fig. 8). Residual bodies were commonly detected (Fig. 11).

Another frequent finding was the presence of large bundles of cytoskeletal filaments together with abundant

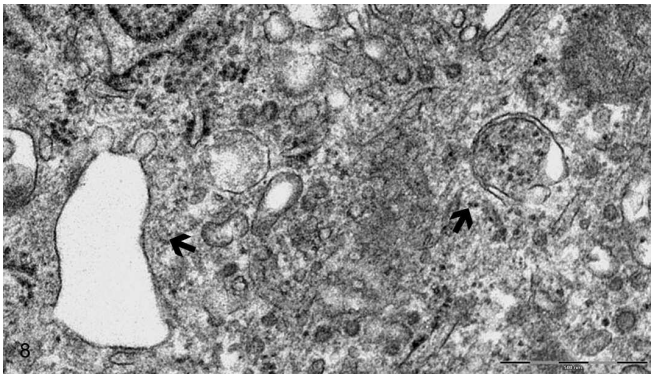


Fig. 8. On the left, endocytic vesicles fusing with an early endosome (arrow). On the right, an early autophagosome surrounded by an incomplete double membrane and containing subcellular structures (arrow). Scale bar: 500 nm.

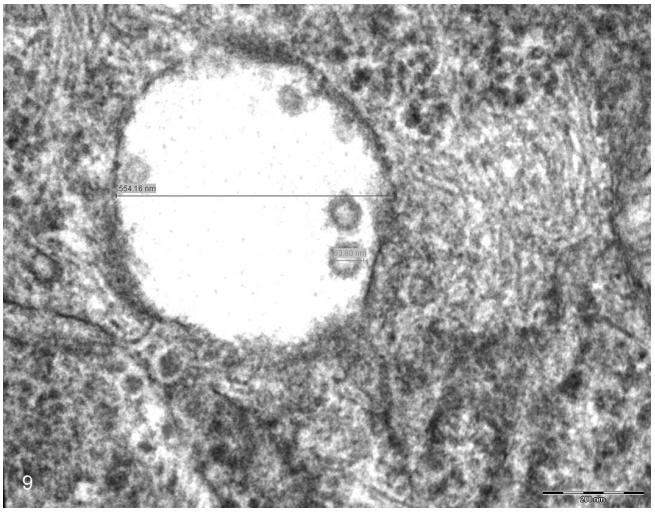


Fig. 9. A late endosome in a early phase of maturation, measuring 554.16 nm and containing 30 to 70 nm wide intralumenal small vesicles. Scale bar: 200 nm.

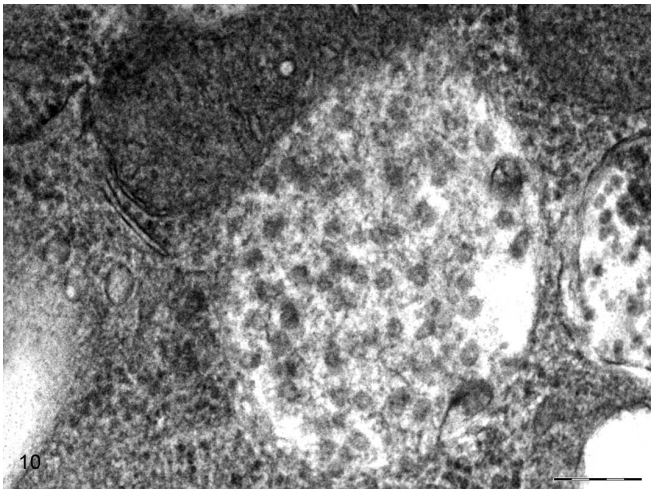


Fig. 10. A mature multivesicular body populated by a great number of intralumenal vesicles. Scale bar: 200 nm.

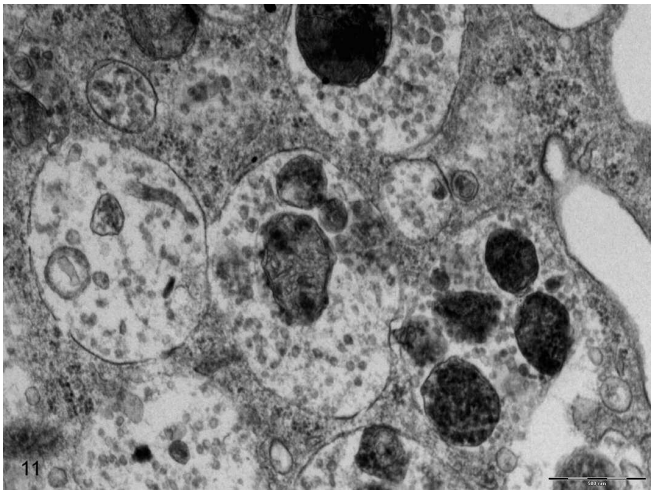


Fig. 11. The electronmicrograph represents several endolysosomes appearing as large vacuolar inclusions with a heterogeneous content: late endosome derived vesicles are mixed with electron-dense material. In the bottom right corner of the figure, a group of residual bodies. Scale bar: 500 nm.

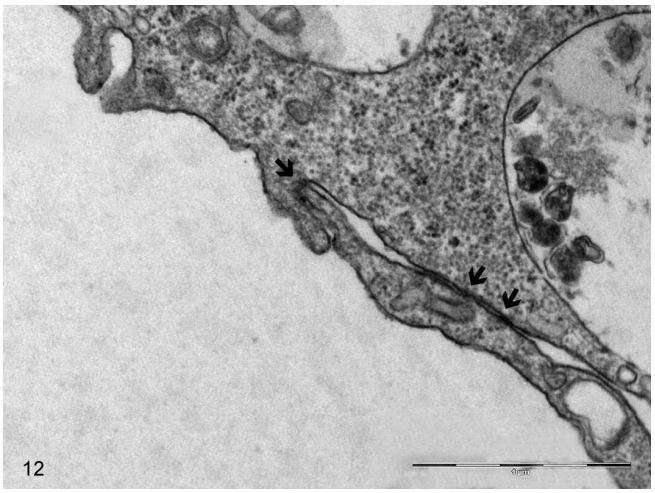


Fig. 12 A thin cell process is inserted into a deep plasma membrane invagination of a contiguous cell. Arrows point out intercellular contacts. Scale bar: 1 μm.

microtubules. The filaments were characteristically organized beneath the cellular membrane, all around the nucleus and randomly in the jaloplasm. Lipid droplets were rarely seen.

Finally, the cells were often interconnected by adhesion contacts found both between cell bodies and at the level of filopodia contacting other cells. These cell processes, extremely variable in length and with a tentacle-like appearance, were characterized by small points of adhesion and were either in contact with each other or inserted into deep plasma membrane invaginations (Fig. 12).

Discussion

Mesenchymal stem cells are considered to be a well accepted research tool usable to study a variety of physiological and pathological conditions, including genetic disorders, differentiation and early developmental processes, drug effects, etc. Above all that, they are of special interest for their clinical potential in tissue repair and gene therapy. Over the last few years, a large number of investigations have given rise to rapid advances in the characterization of MSCs derived from different sources, including adipose tissue. However, despite the growing interest concerning these cells and their use in cell-based therapies, there is serious lack of research on their ultrastructural features. Stem cell stability in culture conditions, meaning the maintenance of basic morphological and functional features ("stemness"), is one of the most debated issues regarding stem cells. Stemness indicators and, among them, morphological features, are essential to discriminate stem cells able to address transplantation procedures from defective cell lines unusable for further applications.

In this work we discuss the establishment of mesenchymal stem cell cultures from equine adipose tissue and describe their morphological features at the ultrastructural level. In order to achieve a good level of homogeneity, cells were maintained in culture until passage 3 prior to morphological examination.

To confirm the multilineage potential of Ad-MSCs, cells were first analyzed for their capacity to differentiate toward adipogenic, osteogenic and chondrogenic lineages. In fact, together with their ability to adhere and proliferate on a plastic surface (adhesiveness and selfrenewing), multipotency is one of the defining characteristics of MSCs and a crucial requisite to identify a putative MSC population. Results obtained in differentiation assays demonstrated that equine Ad-MSCs have the capacity to develop, *in vitro*, into multiple terminally differentiated mesenchymal phenotypes.

In addition, the expression of CD90 surface antigen, a reliable marker of MSCs, was further proof of their nature.

Morphological evaluations were performed on cells scraped from culture wells; as observed by Li et al. (2008) this technique is preferable to the use of trypsin because it preserves the original morphology and the subcellular spatial organization better, enabling to relate of morphological findings to functional observations.

The cytoplasm of AdMSCs was filled with numerous vacuoles. A large number of "unidentified vacuoles" was described by Colter et al. (2001) while Pasquinelli et al. (2007a,b) described a "multiloculated peripheral appearance" and the presence of autophagic vacuoles inside amnion MSCs. As proposed by Raimondo et al., (2006) some of these vacuolar elements consisted of various-sized dilated membranous structures made up of rough endoplasmic reticulum and containing an amorphous material. Dilation of the RER has been documented to occur in normal and pathologic situations and reflects an elevated protein synthesis. "Hyperfunction" of AdMSCs was confirmed by their richness in free ribosomes and by their typical euchromatic nuclei with a deeply indented membrane, expression of an intense synthetic and metabolic activity. Apart from RER dilation, the large amount of the vesicular and vacuolar structures we observed was interpreted as being a remarkable development of the endosomal apparatus. The great abundance of primary endocytic vesicles, early endosomes, late endosomes, endolysosomes, and dense bodies, was evocative of a considerable endocytotic activity due to the intense traffic of molecules from the culture medium to the cytoplasm. Golgian vesicles, too, appeared very numerous and often quite large, contributing to give the cytoplasm a vacuolar appearance.

The occurrence of autophagocytosis was viewed as the expression of the physiologic replacement of subcellular structures caused by the intense turn over.

The observation of numerous pseudopodia and filopodia is not surprising in adhering and migrating

cells. Instead, the particular interaction between such processes and adjacent or distant cells and between cell bodies should be emphasized. As documented by Wuchter et al. (2007), these points of contact are able to establish cell to cell connections and are thought to be implicated in recognition and in cellular interactions.

Taken together, the above ultrastructural results revealed that the equine AdMSCs described herein have the characteristics of undifferentiated cells with an intense synthetic and metabolic activity.

Research efforts focused on further identification of the distinctive morphological features of MSCs could promote a better understanding of their molecular, biological and physiological characteristics as well as their behaviour after transplantation.

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