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Horse adipose-derived mesenchymal stromal cells constitutively produce membrane vesicles: a morphological study

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Summary. Mesenchymal stromal cells (MSCs) are multipotent somatic cells that can differentiate into a variety of mature cell types. Over recent years, their biological *in vitro* and *in vivo* properties have elicited great expectations in the field of regenerative medicine, immunotherapy and tumour treatment. An increasing number of experimental observations suggest that their biological effects are probably related to a paracrine mechanism via the release of trophic factors and cytokines as well as through the production of membrane vesicles (MVs). These are nanometric membrane-bound structures, comprising shedding vesicles (SV) and exosomes (Ex), that enclose and transfer signalling molecules to target cells.

We hypothesized that MVs may be implicated in the biological effects of MSCs from horse adipose tissue (E-AdMSCs), a type of MSC that has been extensively studied in recent years for its remarkable efficacy in tissue regeneration.

By means of electron microscopy, we ascertained, for the first time, that equine adipose-derived MSCs constitutively produce MVs (E-AdMSCs). The analysis of MVs separated by ultracentrifugation allowed us to describe their general morphological features. Through the examination of cell monolayers by TEM, additionally, we distinguished the different pathways of SV and Ex formation, demonstrating that both fractions are produced by E-AdMSC.

The accurate description of MV heterogeneous morphological characteristics led us to emphasize the possible implications of the relationship between *different morphologies versus different functions*.

The data presented in this paper has an additional value, as they can be noteworthy for horses as well as for other mammalian species, including humans.

Key words: Membrane vesicles, Microvesicles, Exosomes, Microparticles, Horse mesenchymal stromal/stem cells

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Introduction

Mesenchymal stromal cells (MSCs) are multipotent somatic cells that can differentiate into a variety of mature cell types. The isolation of MSCs from many adult human and animal tissues (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) and the discovery of their wide biological potential have greatly promoted the study of their influence on numerous physiological and pathological processes. MSCs have proved to be able to migrate to body sites affected by pathological processes (homing) (Sohni and Verfaillie, 2013); they additionally display immunosuppressive and immune-modulatory properties (Stagg and Galipeau, 2013) and modulate the inflammatory response (Nair and Saxena, 2013) and tumour progression (Barcellos-de-Souza et al., 2013). Furthermore, their capacity to deliver genes and to

uptake and release pharmacological molecules (Pessina et al., 2013) has enhanced the spectrum of prospective therapeutic applications.

Until recently, the effects of MSCs *in vivo* were mainly interpreted as the result of binomial proliferation-differentiation (Kopen et al., 1999; Mezey et al., 2000; Caplan and Bruder, 2001). This paradigm has been recently reformulated due to the observation that MSCs act through different mechanisms that probably include – but are not limited to – integration and differentiation in the target tissue cells. MSC biological effects, in fact, depend upon a multitude of signals and complex interactions with resident healthy, damaged or transformed cells that range from direct cell-cell interaction to production of a great variety of signalling molecules (Liang et al., 2014). The observation that these latter can be wrapped up and delivered inside membrane-bound vesicles (MVs) has highlighted the interest towards these nanometric structures that can be responsible, at least in part, for the paracrine action of MSCs (Lavoie and Rosu-Myles, 2013). It has been reported that MVs are produced through two different pathways: the outward budding of the cell surface followed by detachment and release in the extracellular environment of shedding vesicles (SVs); or the inward budding of the outer membrane of multivesicular bodies (MBs) and the formation of exosomes (Ex), released into the extracellular *milieu* through the fusion of MBs with the plasma membrane (Gyorgy et al., 2011).

MVs exert a wide spectrum of functions depending on their content, which, in turn, is related to the type and to the functional state of the cells from which they derive. Protein, lipids and RNAs may be packaged inside MVs; these can variously interact with neighbouring or distant cells determining multiple effects. A growing number of studies demonstrate that MVs act as a transcellular delivery system that operates by a dual mechanism: by changing the secretome of target cells through the transfer of RNAs or, alternatively, by transferring cytokines or growth factors able to modulate the behaviour of target cells (Ratajczak et al., 2006).

Recent studies have demonstrated that MV release occurs from a wide variety of cell types (Raposo and Stoorvogel, 2013). MVs, in addition, have been collected from several biological fluids, including peripheral blood (Caby et al., 2005), urine (Pisitkun et al., 2004), semen (Park et al., 2011; Alberts et al., 2012), saliva (Ogawa et al., 2011), breast milk (Admyre et al., 2007), amniotic fluid (Asea et al., 2008), ascites fluid (Andre et al., 2002), cerebrospinal fluid (Vella et al., 2007) and bile (Masyuk et al., 2010), and have been employed as prognostic and diagnostic indicators/markers in different pathologic conditions.

MVs produced by MSCs (MSC-MVs) have been evaluated in several studies (Biancone et al., 2012; Bruno et al. 2009, 2012; Gatti et al., 2011). Even if much remains to be understood about their biogenesis and mechanism of action, several authors have reported that MSC-MVs may have a similar efficacy as donor cells when used in tissue repair and in anti-cancer therapy (Tetta et al., 2011; Baglio et al., 2012).

In recent years, MSCs from horse adipose tissue (E-Ad-MSCs) have been extensively studied for their efficacy in tissue regeneration. In this species, studies regarding the kinetics of growth, the morphological characteristics and the efficacy *in vivo* have been performed, but in-depth investigations concerning the mechanism of action are lacking.

The aim of this work was to substantiate the hypothesis that adipose-derived MSCs may act through a mechanism that involves MVs production. For this reason, we demonstrated by electron microscopy that they constitutively produce MVs (E-Ad-MSCs-MVs) and we surveyed the most relevant morphological features evidenced by electron microscopy.

Materials and methods

MSC isolation and culture

Tissue samples were collected under general anaesthesia from subcutaneous fat of 1-year-old donor horses hospitalized for abdominal surgery. The samples were washed extensively with sterile PBS supplemented with 200 U/ml penicillin, 200 mg/ml streptomycin and 12.5 mg/ml amphotericin B (Sigma-Aldrich, St. Louis, Mo, USA) to remove debris and red blood cells. Tissue was then finely minced and digested with 0.075% collagenase type I (Worthington Biochemical, Lakewood, NJ, USA) at 37°C for 45 minutes and centrifuged at 600 g for 10 minutes to obtain a pellet. The supernatant was discarded, while the precipitated cell fraction (SVCF) was seeded in tissue culture flasks with DMEM (Gibco, Gaithersburg, MD, USA), 10% FBS (Gibco, Gaithersburg, MD, USA), 100 U/ml penicillin and 100 mg/ml streptomycin. After 72 h of incubation at 37°C with 5% CO_2, the non-adherent fraction was discarded, while the adherent cells were expanded until they achieved 80% confluence. At this time, they were detached using 0.5 mM EDTA/0.05% trypsin (Gibco, Gaithersburg, MD, USA) and plated at a density of 5-10x10^3 cells/cm^2 (Passage 1). The cells (E-AdMSCs) were maintained in the described medium until passage 3. Then, they were prepared for MV isolation and for electron microscopy (EM) observation.

MV isolation and preparation for EM

E-AdMSCSs at passage 3 were grown to 90% confluence in three T75 flasks (about 8x10^6 cells/flask) and incubated for 72 hours in FBS-free DMEM with 0.5% BSA. The culture supernatant (total volume: 90 ml) was centrifuged at 2000 g for 20 min to remove debris and dead cells. Cells were detached with trypsin-EDTA and a trypan blue dye exclusion test was used to determine the number of viable cells present in the suspension. Viable cells were about 98% of the total cells. Cell-free supernatant was then centrifuged at 100,000g for 1 h at 4°C (Beckman-Coulter ultra-centrifuge XL-100K), washed in 0.1 M cacodylate buffer

(CB) pH 7.3 and submitted to a second ultracentrifugation in the same conditions. The MV pellet was finally suspended in 200 µl of CB.

MV suspension (20 µl) was placed on Parafilm. A formvar-coated copper grid (Electron Microscopy Sciences, Hatfield, Pa, USA) was gently placed on the top of each drop for about 60 minutes in a humidified chamber. Grids were then washed in CB pH 7.3 and finally fixed by depositing them for 10 minutes on a drop of 2.5% glutaraldehyde (Fluka, St. Louis, MO, USA) in CB placed on Parafilm. After washing in CB, MVs were contrasted with 2% uranyl acetate. The grids were then air dried and observed under a Philips EM 208 transmission electron microscope (TEM) equipped with a digital camera (University Centre for Electron Microscopy (CUME) - Perugia).

For scanning electron microscopy (SEM), MVs suspended in CB were allowed to adhere to formvar-coated copper grids and fixed as described for TEM. The preparations were attached on metal stubs, coated with chrome to a thickness of 10 nm and examined with a ZEISS - LEO 1525 (Laboratorio Universitario di NAnomateriali - University of Perugia).

Electron microscopy of cell monolayers

For TEM observation, MSC monolayers were fixed with 2.5% glutaraldehyde in 0.1 M CB 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 CB, post-fixed in 2% osmium tetroxide, dehydrated in graded ethanol up to absolute, pre-infiltrated and embedded in Epon 812. Ninety-nm thick sections were mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined under a Philips EM 208 TEM equipped with a digital camera (CUME - Perugia).

For SEM observation, cells were grown on glass coverslips, fixed with 2.5% glutaraldehyde in 0.1 M (CB), pH 7.3, for 2 h at room temperature and dehydrated in a graded series of ethanol up to absolute. The coverslips were mounted on metal stubs and coated partly with gold to a thickness of 15 nm for examination with Philips XL30 Scanning Electron microscope (CUME - Perugia) and partly with chrome to a thickness of 10 nm for examination with ZEISS - LEO 1525 (Laboratorio Universitario di NAnomateriali - University of Perugia).

Results

Characterization of E-AdMSCs MVs by TEM and SEM (Table 1)

When observed by TEM, the pellet obtained by ultracentrifugation of E-AdMSC supernatants revealed the presence of MVs, mainly round-shaped, ranging in

Table 1. Main distinctive features of the investigated structures.

Types of vesicles	ISOLATED MV	WHOLE CELLS
Exosomes (Ex)	Round-shaped Indistinguishable from SV	Round-shaped, about 30-100 nm in diameter. Observed as aggregates in the vicinity of the source cells.
Shedding vesicles (SV)	Round-shaped Indistinguishable from Ex	Round-shaped, about 60 nm to 1 µm in diameter. Slightly to moderately electron-dense, sometimes showing a cytoplasm-like texture or a convoluted membrane.
Membrane vesicles (MV)	Exosome and shedding vesicles together. Round-shaped, 30 to 200 nm in diameter	Exosome and shedding vesicles together.
Macrovesicles	Not detected	Large extracellular vesicles (500 nm to 2 µm and more in diameter) containing smaller vesicles.
Multivesicular bodies	Not detected	Large vacuoles (at least 500 nm in diameter but often up to 2 µm) containing 30 to 100 nm and sometimes wider intra-lumenal vesicles, whose number increase with their gradual maturation. Numerous inside the cells, with different degrees of maturation.

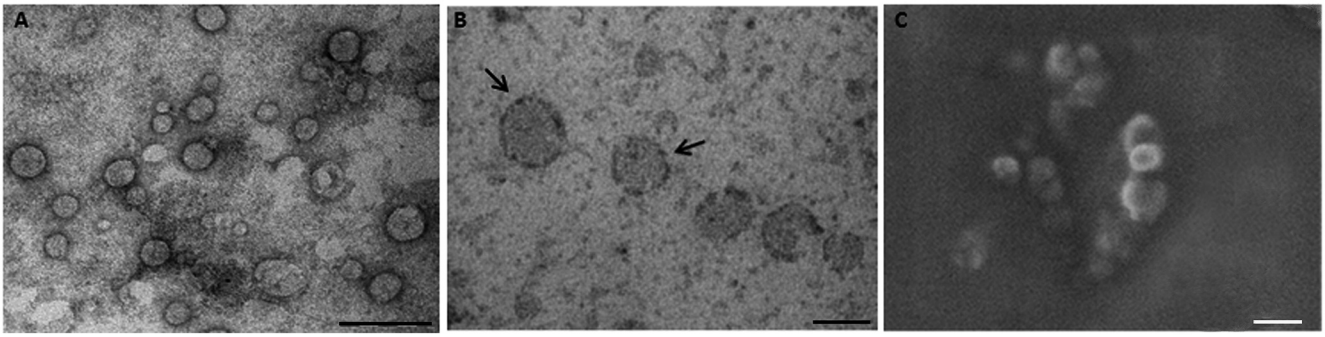

Fig. 1. Electron micrographs showing MVs isolated by ultracentrifugation from E-AdMSC supernatants. **A.** Round-shaped MVs measuring from 30 to 200 nm. The content is uniformly and moderately electron-dense. TEM. **B.** Arrows point to the membrane that is sometimes broken, probably as a result of the isolation procedure. TEM. **C.** Isolated MVs observed by SEM. Acceleration voltage, 1 kV. Scale bar: A, 200 nm; B, C, 100 nm.

size from 30 to 200 nm. They were mainly isolated or aggregated in small clusters and showed a peripheral limiting membrane surrounding a homogeneous electron-lucent to moderately electron dense content. The external margin was occasionally interrupted, probably due to the isolation procedure (Fig. 1).

By SEM, isolated MVs revealed the same characteristics, showing a round shape and overlapping dimensions (Fig. 1).

Through the observation of cell monolayers by electron microscopy, we could detect different phases of MV formation and release. By TEM, numerous variably sized SVs, constitutively budding from the E-AdMSC surface, were seen (Fig. 2). They ranged in size from 60 nm to 1 μm and their boundary displayed the typical features of cell membranes showing an electron-dense trilaminal structure. SV content was generally slightly to moderately electron-dense (Fig. 3); however, vesicles showing a cytoplasm-like texture were occasionally seen (Fig. 4).

The sporadic detection of strongly electron-lucent vesicles located close to the cell membrane and exhibiting convoluted membrane has been documented (Fig. 5).

Concerning Ex detection, it should be emphasized that late endosomes, also referred to as multivesicular endosomes or multivesicular bodies (MVBs), were very numerous inside E-AdMSCs and showed a typical

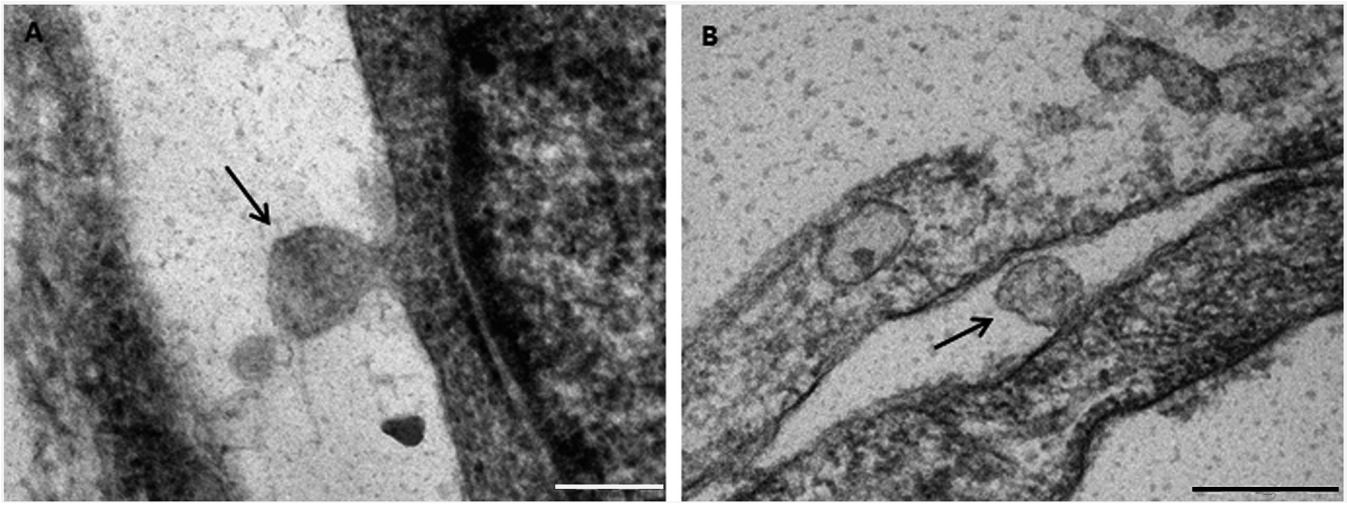

Fig. 2. The figures show a vesicle shedding from the cell surface **(A)** and another one just extruded by the cell of origin **(B)**. TEM. Scale bar: 200 nm.

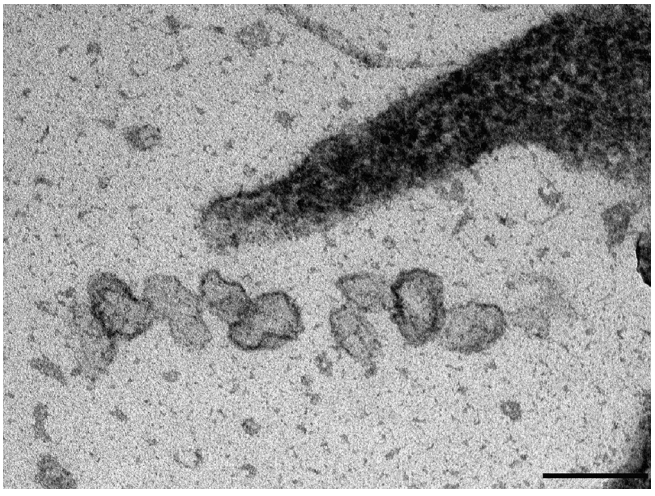

Fig. 3. The picture shows a group of MVs lying near the parental cell body and displaying a slightly electron-dense content. TEM. Scale bar: 200 nm.

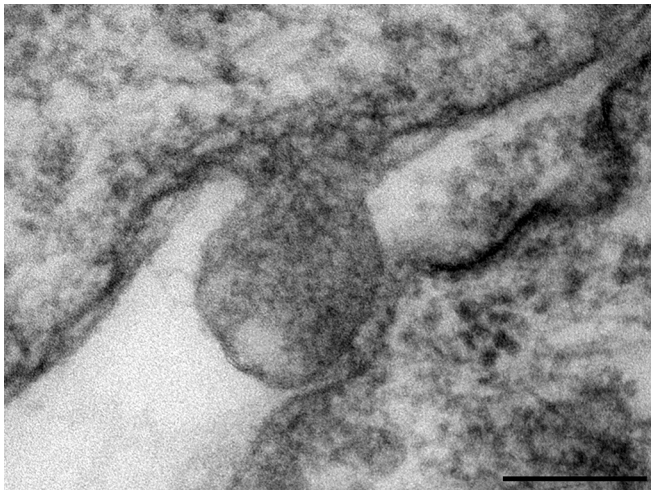

Fig. 4. The figure shows a shedding vesicle characterized by a cytoplasm-like texture. TEM. Scale bar: 200 nm.

multiple-compartment structure. They appeared as large vacuoles (at least 500 nm in diameter but often up to 2 μm) containing 30 to 100 nm and wider intra-lumenal nanovesicles whose number increased with the gradual maturation of the endosome (Fig. 6).

The exact time of exosome release, which is the fusion of MV body membrane with cell membrane and exosome discharge in the extracellular environment, was never detected. However, MVs clustered near the cell membrane and compatible with the recent extrusion of exosomes by the multivesicular body were sometimes observed (Fig. 7).

The presence of membrane-bound "macrovesicles" containing microvesicles in the extracellular environment was quite frequently observed (Fig. 8).

By SEM, a "crown" of MVs surrounding the parental cell was frequently seen. At high magnification, the cell surface appeared extremely irregular for the presence of a myriad of nascent, maturing and detaching vesicles (Fig. 9). Distinguishing SVs and Ex was not possible by SEM observation except by their dimensions.

Discussion

Over recent years, the biological *in vitro* and *in vivo* properties of MSCs have aroused great expectations for their possible application in tissue regeneration,

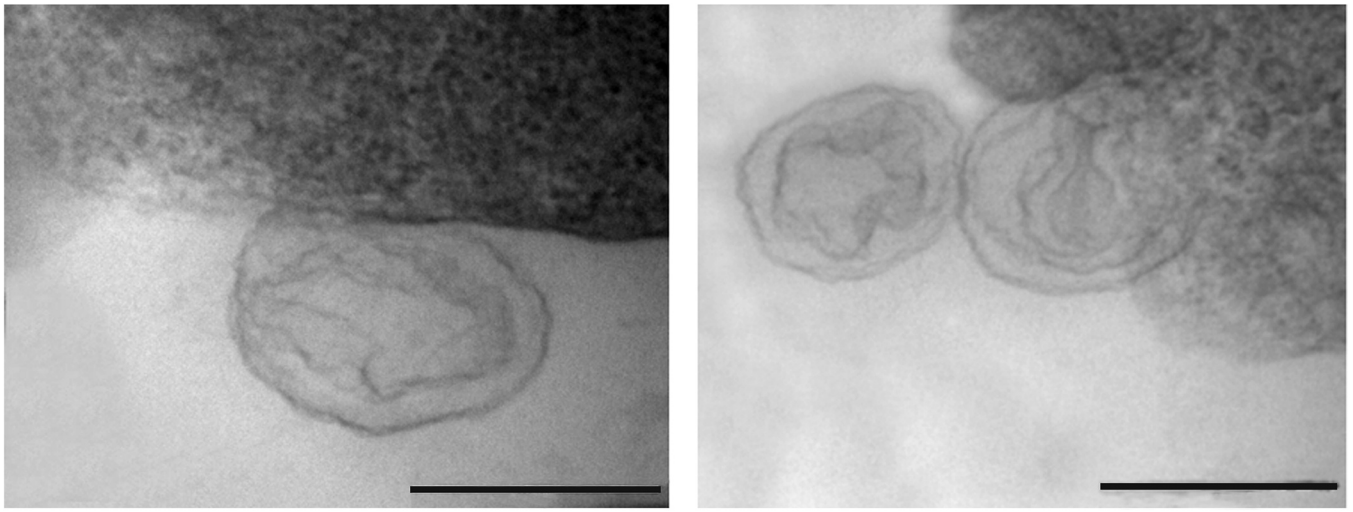

Fig. 5. Membrane vesicles located near the cell membrane displaying a peculiar convolution of limiting membrane. TEM. Scale bar: 500 nm.

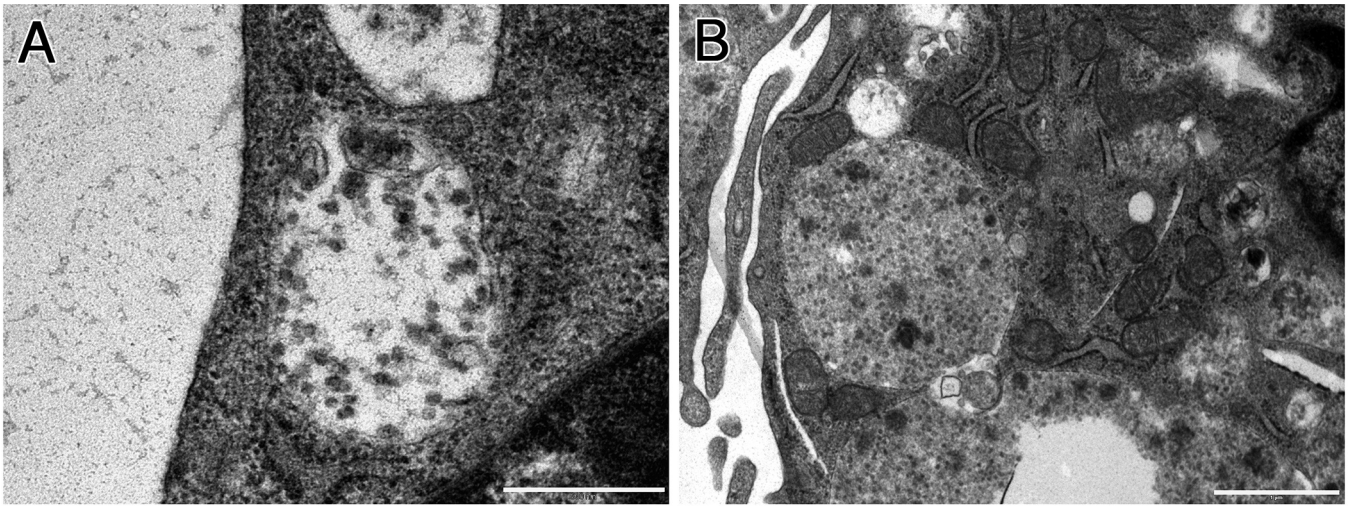

Fig. 6. Maturing multivesicular bodies containing exosomes of various sizes. TEM. Scale bar: A, 500 nm; B, 1 μm.

immunotherapy and tumour treatment. An increasing number of experimental observations suggest that their biological effects are carried out through a paracrine mechanism via the production and release of signalling molecules in a soluble form or embedded inside membrane vesicles (MVs).

MVs are nanometric membrane-bound structures produced by many cell types. Initially considered as being cellular debris, it has now been ascertained that MVs act as carriers of signalling molecules among cells. It has been demonstrated by several authors that MVs may have similar effects to donor cells *in vitro* and *in vivo* and that they are constitutively produced by most cells, including MSCs (Ratajczak et al., 2006; Bruno et al., 2009, 2012; Gatti et al., 2011; Gyorgy et al., 2011; Lee et al., 2012).

By means of electron microscopy, we herein demonstrated that E-AdMSCs constitutively produce MVs. In addition, we provided, for the first time, an extensive description of MVs originating from MSCs observed by electron microscopy. The analysed MVs were sedimented at 100,000 g thereby obtaining a heterogeneous mixture of Ex and SVs. Even if Ex and SVs were isolated as a unique fraction and were not separated before observation, the examination of cell monolayers by electron microscopy allowed us to clearly distinguish the different pathways of SV and Ex formation, demonstrating that both fractions are produced by E-AdMSCs. The registered range of MV dimension led us to deduce that particles over 200 nm, described in numerous relevant papers, are quite rare and are probably lost during the isolation procedure.

This observation points to the possibility that some functional characteristics described in a great number of papers and ascribed to Ex or more generally to MVs are actually attributable to a subpopulation of MVs that likely comprises a mixture of Ex and small SVs precipitated by ultracentrifugation.

The presence of aggregates is commonly described in the literature as a consequence of ultracentrifugation and poor specimen purification from the medium protein fraction. In this study, we detected a limited number of clusters, thereby confirming the good quality of the selected isolation protocol.

An interesting observation concerns the heterogeneous electron density of MVs that could be reasonably related to a different content and mechanism of exocytosis. In fact, we observed that while the greater part of MVs was slightly to moderately electron-dense revealing a soluble content, some MVs were characterized by a cytoplasm-like texture. This second feature could be interpreted as a sort of micro-apocrine secretion in which a very small part of the cell pinches off and is delivered into the extracellular environment.

Concerning the quite large MVs that exhibited an extremely electron-lucent content and a convoluted membrane, they probably comprise a category of MVs that, by virtue of their content and of their structure, are quite deformable and are probably even larger than they appear. The possibility that it may be an artifact due to aldehyde fixation cannot be ignored.

The great abundance of multivesicular bodies inside E-AdMSCs, described even in a previuos paper (Pascucci et al., 2010), suggests that an intense membrane vesicle traffic is achieved along both the endocytic and the esocytotic pathways and that Ex strongly contribute to the formation of microvesicular progeny.

Finally, the remarkable detection of membrane-bound macrovesicles containing microvesicles located in the extracellular environment cannot be ignored. To our knowledge, no similar observations have been made before, nor can the traditional principles of transmembrane trafficking easily justify the exocytosis of entire multivesicular bodies. This finding opens new perspectives in the interpretation of intercellular communication pathways mediated by membrane vesicles. It could be speculated that intracellular vesicles enclosing an undefined content are polarized underneath a distinct region of the plasma membrane and are then exocytosed through a sort of micro-apocrine secretion. In this way, the cell could be able to package a homogeneous or heterogeneous cargo intended for a unique destination.

In conclusion, our data provide an extensive description of MVs originating from E-AdMSCs at the fine morphological level. The observed morphological diversity of MVs described in this study strengthens the idea that a variety of morphologies may correspond to a variety of functions, contents and release mechanisms and suggest the necessity of further in depth studies exploring the associated functional implications. In fact, the only morphological approach, even if highly informative, runs the risk of being merely descriptive

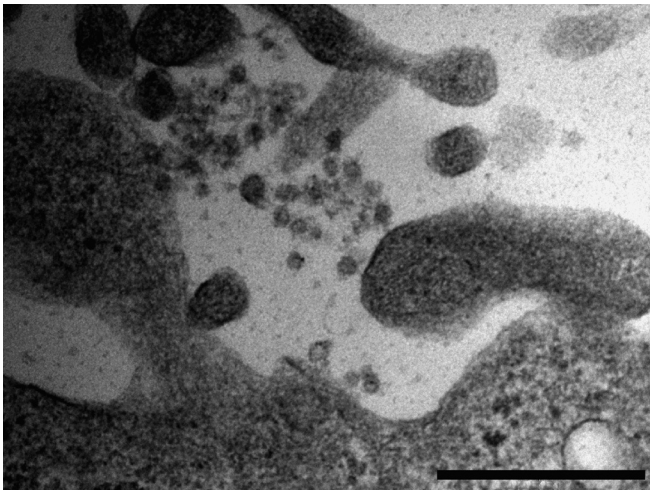

Fig. 7. The figure shows a cluster of MVs located near the cell membrane. They are probably exosomes recently released by a multivesicular body in the extracellular environment. TEM. Scale bar: 500 nm.

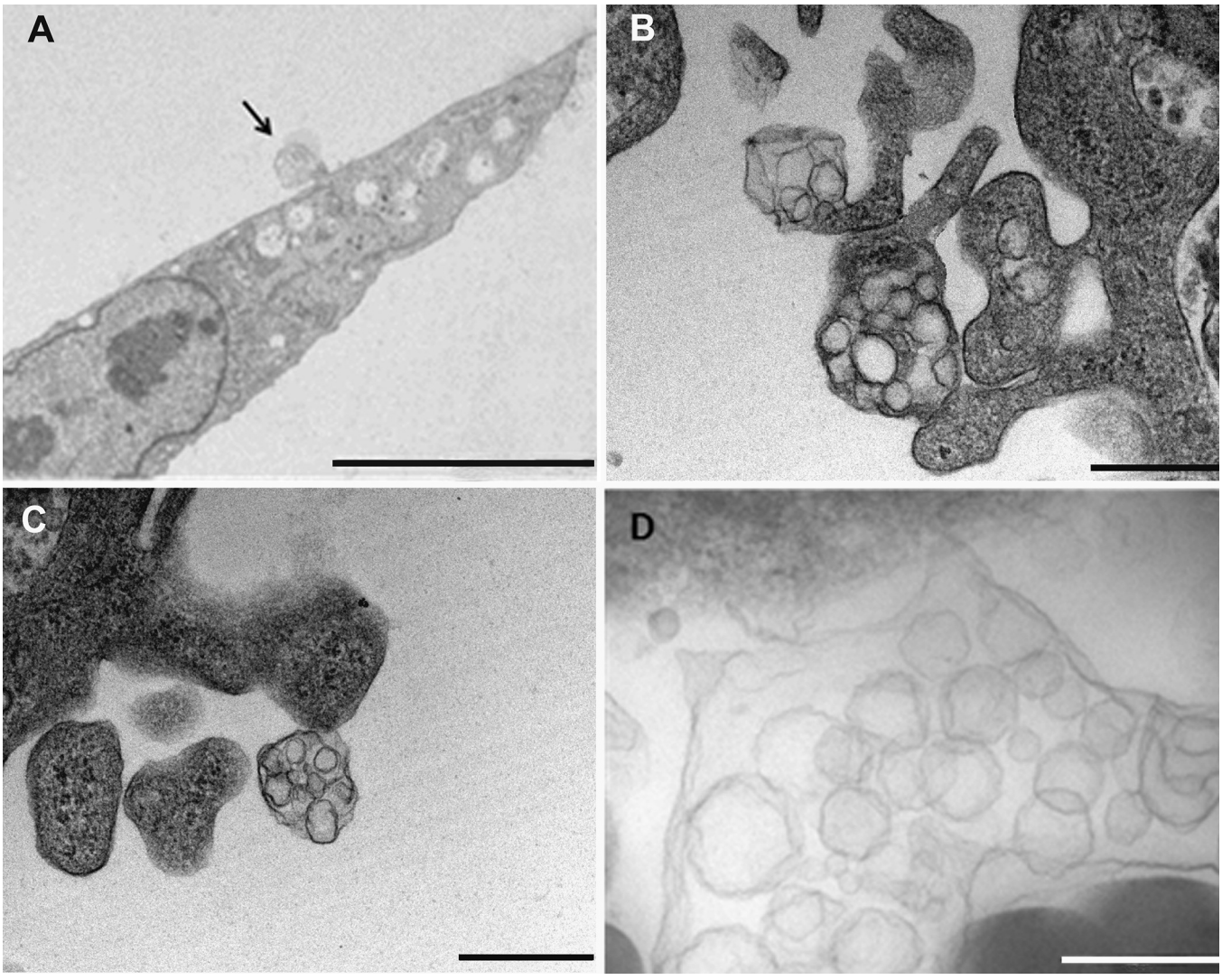

Fig. 8. Membrane-bound "macrovesicles" containing "microvesicles". Scale bars: A, 10 μm; B-D, 500 nm.

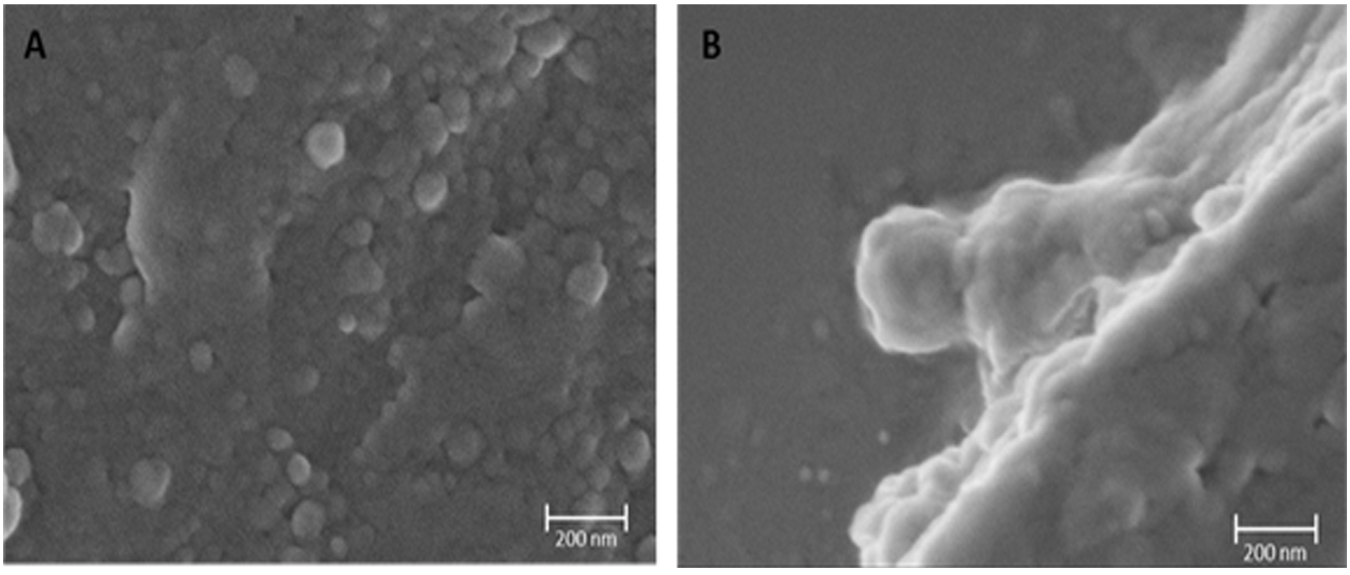

Fig. 9. A. The aspect of the cell surface determined by the presence of maturing vesicles. **B.** A particular SV is detaching from the cell surface. SEM. Scale bar: 200 nm.

and speculative. Therefore, further studies aimed at the investigation of biokinetics, content and functions of the different types of MV described in this paper represent the natural and necessary development of this research work. Additionally, the use of immunoelectron microscopy is mandatory to have an accurate characterization of the heterogeneous mixture of Ex and SVs comprised in whole mount preparations.

It should finally be emphasized that the data presented in this paper may be significant for horses as well as for other mammalian species, including humans. In fact, numerous experimental observations confirm the high analogy of MV bio-kinetics, content and mechanism of action among mammals.

Conflict of interest statement. None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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