

Characterization and potential applications of progenitor-like cells isolated from horse amniotic membrane

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Abstract

The aim of this work was to isolate, for the first time, progenitor-like cells from the epithelial (AECs) and mesenchymal (AMCs) portions of the horse amniotic membrane, and to define the biological properties of these cells. AECs displayed polygonal epithelial morphology, while AMCs were fibroblast-like. Usually, six to eight passages were reached before proliferation decreased, with 13.08 and 26.5 cell population doublings attained after 31 days for AECs and AMCs, respectively. Immunocytochemical studies performed at passage 3 (P3) showed that both cell populations were positive for the expression of specific embryonic markers (*TRA-1-60*, *SSEA-3*, *SSEA-4* and *Oct-4*). Meanwhile, RT-PCR performed at P1 and P5 showed expression of mesenchymal stem/stromal cell markers (*CD29*, *CD105*, *CD44* and *CD166*) with negativity for *CD34* at P1, although this marker began to be expressed by P5. The cells also expressed *MHC-I* at both P1 and P5, but lacked *MHC-II* expression at P1. Both AECs and AMCs demonstrated high plasticity, differentiating *in vitro* toward the osteogenic, adipogenic, chondrogenic and neurogenic lineages. Equine amnion-derived cells could also be frozen and recovered without loss of their functional integrity in terms of morphology, presence of specific stemness markers and differentiation ability, although the renewal capacity was lower than that observed for freshly isolated cells. To investigate potential therapeutic effects and cell tolerance *in vivo*, horse amnion-derived cells were allogeneically injected into three horses with tendon injuries, resulting in a quick reduction in tendon size and ultrasonographic cross-sectional area measurements. These results suggest that horse amnion-derived cells may be useful for cell therapy applications. Copyright © 2011 John Wiley & Sons, Ltd.

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Supporting information may be found in the online version of this article.

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1. Introduction

For the effective application of stem cells in regenerative medicine, the ability to collect a large number of cells, to

harvest the cells inexpensively and non-invasively without risk to the donor and to cryogenically bank and expand the cells *in vitro* are very important considerations (Carlin *et al.*, 2006). In veterinary medicine, therapeutic approaches to treat orthopaedic injuries affecting musculoskeletal tissues, such as tendon and cartilage, which are the most common causes of lost training days or premature retirement in the equine athlete, have been developed using multipotent mesenchymal stem/stromal cells (MSCs) (Smith *et al.*, 2003; Pacini *et al.*, 2007; Fortier

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and Smith, 2008; Guest *et al.*, 2008). To date, horse MSCs have been isolated mainly from bone marrow (BM) (Koerner *et al.*, 2006; Vidal *et al.*, 2006; Kisiday *et al.*, 2008) and adipose tissue (Vidal *et al.*, 2006; Kisiday *et al.*, 2008) but also from peripheral blood (Koerner *et al.*, 2006), umbilical cord blood (Koch *et al.*, 2007; Reed and Johnson, 2008; Berg *et al.*, 2009) and umbilical cord matrix (Hoynowski *et al.*, 2007; Cremonesi *et al.*, 2008; Passeri *et al.*, 2009). Despite the ever-increasing use of cell-based therapies for treating equine orthopaedic problems, questions relating to the optimal cell source, timing and route of treatment for different injuries, as well as long-term safety and efficacy of these treatments, still remain largely unanswered (Berg *et al.*, 2009).

Although BM-MSCs represent the most widely investigated cells for application in veterinary regenerative medicine to date, these cells have a more limited potential than embryonic stem cells in terms of both *in vitro* proliferation ability (about 32 days for expansion between isolation and implantation) and target differentiation capacity (Guest *et al.*, 2010), and do not appear to noticeably improve long-term functionality (Paris and Stout, 2010). In addition, procurement of BM-MSCs from horses requires an invasive BM aspiration procedure, which has been associated with pneumopericardium (Durando *et al.*, 2006). Meanwhile, human BM-MSCs show reduced plasticity and growth with increasing donor age and *in vitro* passage number (Digirolamo *et al.*, 1999; Guillot *et al.*, 2007). Therefore, progenitor cells derived from extra-fetal sources may represent attractive alternative candidates, with the potential to circumvent many of these limitations, opening new perspectives for developmental biology and regenerative medicine.

In human medicine, amniotic membrane (AM) isolated from term placenta is a very attractive source of stem/progenitor cells, due to the fact that AM-isolated cells not only show immunomodulatory properties, but also because this extra-fetal tissue is usually discarded without ethical conflicts after birth, and allows highly efficient recovery of MSCs without the requirement for invasive procedures. Human AM or human amniotic epithelial cells (hAECs) have been shown to survive for prolonged times in immunocompetent animals, including rabbits (Avila *et al.*, 2001), rats (Kubo *et al.*, 2001), guinea pigs (Yuge *et al.*, 2004) and bonnet monkeys (Sankar and Muthusamy, 2003). Recent studies have also demonstrated that human amniotic mesenchymal cells (hAMCs) have immunomodulatory properties, can strongly inhibit T lymphocyte proliferation (Magatti *et al.*, 2008) and show low immunogenicity (Bailo *et al.*, 2004). Moreover, it has been reported that human amnion-derived cells possess multipotent differentiation ability (Ilancheran *et al.*, 2007; Evangelista *et al.*, 2008) and anti-inflammatory functions (Sarugaser *et al.*, 2005).

In veterinary medicine, AM has been used in clinical therapies for ocular surface reconstruction in animal patients, including horse (Ollivier *et al.*, 2006; Plummer, 2009) and dog (Arcelli *et al.*, 2009), as it is avascular and strong, promotes re-epithelialization, decreases inflammation

and fibrosis (Solomon *et al.*, 2005) and modulates angiogenesis (Dua *et al.*, 2004).

Considering that healing requires growth factors that stimulate angiogenesis, mitogenesis and matrix formation, amnion-derived cells could represent an invaluable tool for veterinary cell therapy, in particular when one considers that these cells could display plasticity and reduced immunogenicity.

In this context, the aim of this work was to provide, for the first time, an isolation protocol for horse amnion-derived progenitor cells and to investigate the biological properties of these cells, which have potential clinical utility in equine regenerative medicine. Moreover, we characterized the capability of these cells to differentiate toward the osteogenic, adipogenic, chondrogenic and neurogenic lineages. In addition, we evaluated whether these cells could be frozen, stored and recovered without loss of their functional integrity in terms of morphology, renewal capacity, differentiation and presence of specific stemness markers. Finally, we investigated whether horse amnion-derived cells could be tolerated and exert beneficial effects *in vivo* when allogeneically transplanted into horses with tendon injuries.

2. Materials and methods

2.1. Amnion collection

Allanto-amniotic membranes were obtained at term of normal pregnancies and after vaginal delivery from three horses. Portions of allanto-amnion were kept at 4 °C in phosphate-buffered saline (PBS; EuroClone, Milan, Italy) with 100 U/ml penicillin–100 µg/ml streptomycin (Sigma Chemical, Milan, Italy) and amphotericin B (Sigma) and were processed within 12 h. The AM was stripped from the overlying allantois (Figure 1A) and cut into small pieces (about 9 cm² each) before enzymatic digestion.

2.2. Isolation of amniotic epithelial and mesenchymal cells

Isolation of amnion-derived cells was performed as reported by Soncini *et al.* (2007), with some modifications. Briefly, amnion fragments (about 12 g for an extension of 630 cm²) were incubated for 9 min at 37 °C in PBS containing 2.4 U/ml dispase (Becton Dickinson, Milan, Italy). After a resting period (5–10 min) at room temperature in high-glucose Dulbecco's modified Eagle's medium (HG-DMEM; EuroClone) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma) and 2 mM L-glutamine (Sigma), the fragments were digested with 0.93 mg/ml collagenase type I (Sigma) and 20 µg/ml DNase (Roche, Mannheim, Germany) for approximately 3 h at 37 °C. The amnion fragments were then removed, and mobilized cells were passed through a 100 µm cell strainer (Sigma) before being collected by centrifugation at 200 × g for 10 min. We termed these cells 'amniotic mesenchymal

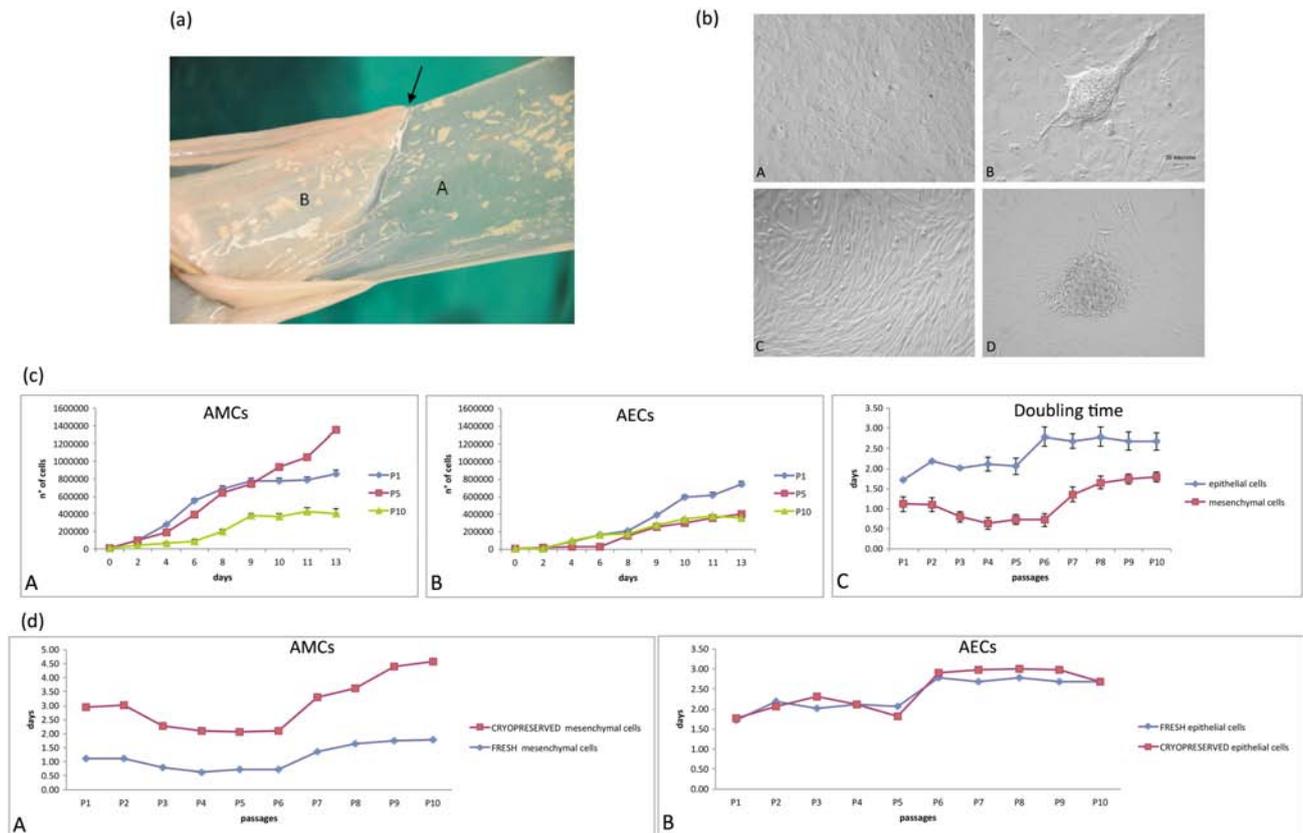


Figure 1. (a) Amniotic membrane (A) was stripped from the overlying allantois (B). (b) Cell morphology. Monolayer of AECs (A) and AECs with cluster (B); magnification, $\times 20$; monolayer of AMCs (C) and AMCs with small cluster (D); magnification, $\times 20$; scale bar = $20 \mu\text{m}$. (c) AMCs (A) and AECs (B) growth curve at passages 1 (P1), 5 (P5) and 10 (P10); (C) doubling times at different passages during cell culture for both AECs and AMCs. Asterisks represent doubling time means statistically different with respect to P1. (d) Cell population doubling comparison between fresh and cryopreserved mesenchymal (A) and epithelial (B) cells

cells' (AMCs). Meanwhile, the collagenase-undigested amnion fragments were further incubated with 0.25% trypsin (Sigma) at 37°C for 2 min in order to obtain amniotic epithelial cells (AECs), which were collected by filtration and centrifugation as described above.

2.3. Cell culture

Cultures were established in HG-DMEM supplemented with 10% FBS, 10 ng/ml epidermal growth factor (EGF; Sigma), 1% penicillin (100 UI/ml)–streptomycin 100 $\mu\text{g}/\text{ml}$, 0.25 $\mu\text{g}/\text{ml}$ amphotericin B and 2 mM L-glutamine. The number of viable cells was counted by the trypan blue dye exclusion method (Sigma), using a Burker chamber. For maintenance of cultures, cells were plated at up to 1×10^5 cells/ cm^2 and incubated at 38.5°C in a humidified atmosphere (90%) with 5% CO_2 . To remove non-adherent cells, the medium was replaced for the first time after 72 h, and then changed either twice per week thereafter or according to the experiment requirements. Adherent cells were detached with 0.05% trypsin–EDTA (EuroClone) just prior to reaching

confluence (80%) and then reseeded for culture maintenance. The cells were expanded for 10 passages, which was the last time point included in our study.

2.4. Proliferation assays

To obtain cell-proliferation growth curves at passages 1, 5 and 10 (P1, P5 and P10), 9×10^3 AMCs or AECs were plated into six-well tissue culture polystyrene dishes (EuroClone). Every 2 days, through 13 days of culture, one well of each plate was trypsinized. The total number of live cells was obtained at each time point by staining with the trypan blue dye exclusion method. The procedure was repeated with cells isolated from three separate AMs. Doubling time of the AMCs and AECs for passages 1–10 was determined by seeding 9×10^3 cells into six-well tissue culture polystyrene dishes. Cells were trypsinized every 4 days, counted and replated at the same density. Mean doubling time was calculated from day 0 to day 4 for three separate AMs. The mean of population doublings (PD) was obtained for each passage

according to the formulae $CD = \log(Nc/No)/\log_2$ and $PD = CT/CD$, where CD represents cell doubling, Nc represents the number of cells at confluence, No represents seeded cells and CT represents the culture time. Data representative of three independent experiments were reported.

2.5. CFU assays

Colony-forming unit (CFU) assays were performed at P0 on freshly isolated cells at different densities (100, 250, 500 and 1000 cells/cm²). Cells were plated in six-well plates and cultured in 5% CO₂ and 90% humidity at 38.5 °C for 2 weeks in HG-DMEM-supplemented medium. Then, colonies were fixed with 4% formalin and stained with 1% methylene blue (Serva, Heidelberg, Germany) in 10 mM borate buffer, pH 8.8 (Fluka BioChemika, Buchs, Switzerland) at room temperature, and washed twice. Colonies formed by 16–20 nucleated cells were counted under a BX71 microscope (Olympus, Japan).

2.6. Differentiation assays

Cells at P3 were seeded at a density of 3×10^3 /cm² for all differentiation studies. Osteogenic differentiation was assessed by incubating cells for up to 3 weeks at 38.5 °C under 5% CO₂ in modified Romanov *et al.* (2003) medium, composed of HG-DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2 mM L-glutamine, 10 mM β-glycerophosphate (Sigma), 0.1 µM dexamethasone (Sigma) and 250 µM ascorbic acid (Sigma) (Romanov *et al.*, 2003). Non-induced control cells were cultured for the same time in standard control medium (HG-DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2 mM L-glutamine). Osteogenesis was assessed by conventional von Kossa staining, using 1% silver nitrate and 5% sodium thiosulphate, which allowed detection of calcium deposits.

For adipogenic differentiation, near-confluent cells were cultured through three cycles of induction/maintenance to stimulate adipogenic differentiation. Each cycle consisted of feeding the amnion-derived cells with supplemented adipogenesis induction medium, followed by culture for 3 days (38.5 °C, 5% CO₂) and subsequent culture for another 3 days in supplemented adipogenic maintenance medium. The induction medium consisted of modified Romanov *et al.* (2003) medium, composed of HG-DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2 mM L-glutamine, 10 µg/ml insulin (Sigma), 150 µM indomethacin (Sigma), 1 µM dexamethasone and 500 µM 3-isobutyl-1-methyl-xanthine (Sigma) (Romanov *et al.*, 2003). The maintenance medium consisted of HG-DMEM supplemented with 10% FBS and 10 µg/ml insulin. Non-induced control cells were cultured for the same time in

standard control medium. Adipogenesis was assessed using conventional oil red O staining (0.1% in 60% isopropanol) to visualize lipid droplets.

Chondrogenic differentiation was assessed in monolayer culture by incubating cells for 3 weeks in Soncini *et al.* (2007) modified medium, composed of DMEM low-glucose containing 100 nM dexamethasone, 50 µg/ml L-ascorbic acid 2-phosphate, 1 mM sodium pyruvate (BDH Chemicals, Poole, UK), 40 µg/ml proline, ITS (5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite; Sigma) and 5 ng/ml TGF-β3 (Peprovet, DBA, Italy). Non-induced control cells were cultured for the same time in standard control medium. The presence of metachromatic matrix was demonstrated by Alcian blue staining, pH 2.5.

Neurogenic induction was performed by culturing cells for 24 h in preinduction medium consisting of HG-DMEM, 20% FBS and 1 mM β-mercaptoethanol (Sigma) (Mitchell *et al.*, 2003; Seo *et al.*, 2009), with neural induction then performed by switching to a medium composed of DMEM plus 2% FBS, 2% dimethylsulphoxide (DMSO; Sigma) and 200 µM butylated hydroxyanisole (Sigma) for 3 days (Woodbury *et al.*, 2000). Non-induced control cells were cultured for the same time in standard medium. Neurogenic differentiation was demonstrated by conventional Nissl staining (0.1% cresyl violet solution), which showed increasing ribosomes.

2.7. Cryopreservation

Cells were frozen at P0 in HG-DMEM supplemented with 50% FBS and 10% DMSO for 6 months at –80 °C. After thawing, some cells were used for population doubling studies, while other cells were expanded until P3 for immunohistochemical studies or to evaluate specific MSC marker expression and multipotent differentiation capacity in comparison to freshly isolated cells.

2.8. Histology and immunostaining

2.8.1. Histology

Immediately upon delivery, horse AM samples were collected, sectioned and fixed in 10% formalin for 24 h. After fixation, the tissues were dehydrated in a graded series of ethanols and embedded in paraffin. Serial sections were cut at 4 µm thickness, dewaxed and stained with haematoxylin and eosin (H&E) for histological examination.

2.8.2. Immunocytochemical characterization

AECs and AMCs at P3 were tested for immunoreactivity against Vimentin (mouse monoclonal, clone Vim V9; Dako, Glostrup, Denmark) and PanCytokeratin (mouse monoclonal, clone A1E; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cultured AECs and AMCs were fixed in Cytoscreen solution (Hospitex Diagnostics, Milan, Italy)

for 48 h, and then cytocentrifuged on Superfrost Plus slides (Thermo Scientific, Menzel GmbH & Co., KG, Braunschweig, Germany) and air-dried.

To test the *in situ* immunoreactivity of horse amnion-derived cells, a fragment of AM was fixed in 10% buffered formalin, embedded in paraffin and cut to obtain 4 µm formalin-fixed and paraffin-embedded sections (FFPEs). The FFPEs were mounted on Superfrost Plus slides, dried overnight at 37 °C and further processed for immunocytochemistry.

Briefly, for the immunocytochemistry procedure, slides were dewaxed in xylene (FFPEs only), passed through a graded series of alcohols, and rehydrated in deionized water. Antigen retrieval (FFPEs only) was performed with a citrate buffer at pH 6.0 in a pressure cooker for 20 min at 98 °C, before cooling at room temperature for 20 min. Endogenous peroxidase was exhausted with Dako Real solution (Dako) for 10 min at room temperature. To block non-specific staining, sections were incubated with Dako protein block solution (Dako) for 30 min at room temperature.

Primary antibodies, diluted in TBST (anti-Vimentin, 1/100; anti PanCytokeratin, 1/200), were applied and incubated overnight at 4 °C. Secondary antibody, conjugated with horseradish peroxidase (HRP), was added and incubated for 30 min at room temperature. The peroxidase reaction was developed for 10 min, using diaminobenzidine (DAB) and following the manufacturer's instructions (ImpactDAB, Vector Labs, Burlingame, CA, USA) and blocked with deionized water. Cells were considered positive for Vimentin and PanCytokeratin when the presence of intracytoplasmic stain was observed. Negative controls for the target antigens were performed by replacing the primary antibodies with irrelevant antibodies from the host species in which the immunoglobulins were developed (rabbit immunoglobulins fraction normal X093 and mouse IgG1 X0931; Dako). Negative controls in which the primary antibody was replaced with only a buffer solution (TBST) were also performed.

To test the expression of embryonic (SSEA-3 and SSEA-4 and TRA-1-60) and pluripotent (Oct-4) markers, antibodies were chosen according to results obtained by Hoynowski *et al.* (2007). Primary antibodies were purchased from Abcam (Cambridge, MA, USA), while AlexaFluor-488 conjugated secondary antibodies were from Invitrogen (Carlsbad, CA, USA). All products were used following the manufacturer's instructions.

For immunostaining, cells at P3 were fixed in 3.7% paraformaldehyde (PFA) for 15 min and washed three times in PBS. For Oct-4, after fixation, cells were permeabilized for 10 min at room temperature in 0.4% Triton-X 100 diluted in PBS and washed three times in PBS. After washing, cells were blocked using 2% BSA in PBS for 4 h at 4 °C and then incubated with primary antibodies overnight at 4 °C. After washing three times, cells were incubated with secondary antibodies conjugated to AlexaFluor-488 (1:250 dilution) for 1 h. Finally, for nuclear staining, cells were incubated for 15 min with Hoechst 33342 (1 mg/ml; Sigma) diluted 1:100 in PBS.

Images were captured on a BX 51 microscope (Olympus, Japan). Fibroblasts, collected from skin biopsies from adult horses, were used as negative control.

2.8.3. Alkaline phosphatase staining

To characterize the undifferentiated state of amnion-derived cells, we analysed the level of alkaline phosphatase (AP) expression. The staining was performed according to the instructions of the AP detection kit from Chemicon International (Temecula, CA, USA). Briefly, at P3, cells were fixed with 4% PFA (Sigma) in PBS for 1–2 min, rinsed with rinse buffer, stained with naphthol/fast red violet solution in the dark at room temperature for 15 min, and finally rinsed with rinse buffer. Colonies expressing AP appeared red (undifferentiated cells).

2.9. RNA extraction and RT-PCR analysis

Expression of specific MSC (*CD44*, *CD29*, *CD105* and *CD166*) and haematopoietic (*CD34*) markers was investigated by RT-PCR analysis on undifferentiated cells.

Total RNA was extracted at P1 and P5 from equine AECs and AMCs, using Trizol[®] reagent (Invitrogen), followed by DNase treatment according to the manufacturer's specifications. RNA concentration and purity were measured using a NanoDrop spectrophotometer (NanoDrop[®] ND1000). cDNA was synthesized from 200 ng total RNA, using the iScript retrotranscription kit (Bio-Rad Laboratories, Hercules, CA, USA). Conventional PCR was performed in a 25 µl final volume with DreamTaq DNA Polymerase (Fermentas, St. Leon Rot, Germany). Equine-specific oligonucleotide primers were designed using open source PerlPrimer software v. 1.1.17, based on available NCBI *Equus caballus* sequences or on mammal multi-aligned sequences. Primers were designed across an exon–exon junction in order to avoid DNA amplification. Primers were used at 200 nM final concentration and their sequences are shown in Table 1. *GAPDH* was employed as a reference gene.

For differentiation experiments, total RNA was extracted from undifferentiated (control cells) and from induced amnion-derived cells, and RT-PCR analysis was performed as described above. Equine adult tissues (bone, fat, cartilage and spinal cord) were employed as positive controls for assessing the expression of osteopontin (*OPN*) and osteocalcin (bone γ -carboxyglutamate protein; *BGLAP*) for osteogenesis, peroxisome proliferator-activated receptor- γ (*PPAR- γ*) and adiponectin (*ADIPQ*) for adipogenesis, collagen type II- α 1 (*COL2A1*) and aggrecan (*ACAN*) for chondrogenesis, and glial fibrillary acidic protein (*GFAP*) and nestin (*NES*) for neurogenesis, respectively. Primer sequences are listed in Table 1.

2.10. Cell transplantation *in vivo*

To evaluate their ability to exert beneficial effects *in vivo*, horse AMCs at P3 were allogeneically transplanted into

Table 1. Oligonucleotide sequences used for RT-PCR analysis

Markers	Sequences (5' → 3')	Product size (bp)	Annealing temperature (°C)
Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	S: AGATCAAGAAGGTGGTGAAG A: TTGTCATACCAGGAAATGAGC	168	60
CD34 molecule (<i>CD34</i>)	S: CAGAAAATCCCAGCAAGCTC A: ATAGCAAATGAGGCCCAAGA	207	56
Integrin- β 1 (<i>CD29</i>)	S: CTTATTGGCCTTGCATTGCT A: TTCCCTCGTACTTCGGATTG	184	63
CD44 antigen (<i>CD44</i>)	S: ATCCTCACGTCCAACACCTC A: CTGCCTTTCTGGTGTAGC	165	63
ALCAM (<i>CD166</i>)	S: CCGTTCACTATTTGGATTTGT A: CGTTTCACAGACATAGTTCC	199	55
CD14 molecule (<i>CD14</i>)	S: TTGATCTCAGCTGCAACAGG A: GTGGGGATATCAGGAACCCT	184	56
Endoglin (<i>CD105</i>)	S: AAGAGCTCATCTCGAGTCTG A: TGACGACCACCTCATTACTG	162	56
Major histocompatibility complex I (<i>MHC-I</i>)	S: GGAGAGGAGCAGAGATACA A: CTGTCCTGTTGCGAGTCT	218	55
Major histocompatibility complex II (<i>MHC-II</i>)	S: TCTACACCTGCCAAGTG A: CCACCATGCCTTTCTG	178	55
Osteocalcin (<i>BGLAP</i>)	S: TGAAGACCAGTATCTCTGATGC A: GCTGACTTGTTTCCTGACTG	174	60
Osteopontin (<i>OPN</i>)	S: GTCTGGCAGAGGTGCGACCT A: ATGTGTCAGCCAGCTCGTC	173	56
Peroxisome proliferator activated receptor (<i>PPAR-γ</i>)	S: TGCCCTTCAACGAAATTACC A: TGGAAATGCTCTCATAGTGTGG	176	55
Adiponectin (<i>ADIPQ</i>)	S: GGAGACAGCTACTCCCAAGAT A: GTCCAGTCTTACCTCTCAAACCT	187	64
Aggrecan (<i>ACAN</i>)	S: TCTGCTACACAGGTGAAGAC A: AAGATGGGTTTCACTGTGAG	174	60
Collagen type 2, α 1 (<i>COL2A1</i>)	S: GGAGACTACTGGATTGACCC A: TCCATAGCTGAAGTGAAGC	201	60
Glial fibrillary acidic protein (<i>GFAP</i>)	S: CAGAAGCTCCAGGATGAAACC A: TGGATCTTCTCAAGAACCAGGA	155	58
Nestin (<i>NES</i>)	S: TGCCCTCAGCTTGCAAGAC A: GTGTCTCGAGAGTATCAGGCAAG	134	60

S, sense; A, antisense; bp, base pairs.

three privately owned sport horses with tendon lesions. The ecographic diagnosis was documented by transverse and longitudinal sections. Specifically, as assessed by ultrasound diagnosis, two horses were affected by acute rupture of a forelimb superficial digital flexor tendon (SDFT), and another horse by an acute lesion of a forelimb accessory ligament of the deep digital flexor tendon (AL-DDFT). After obtaining permission from the institutional ethical committee and written consent from the owners, 1 million allogenic cells, diluted in 800 μ l autologous plasma, were injected into the tendon injury of each horse under ultrasonographic guidance. Hair was clipped with a No. 40 blade. Routine aseptic surgical preparation was performed with a chlorhexidine scrub and alcohol. A local anaesthetic ring block (2% Mepivacaine) proximal to the injection site and mild sedation with Detomidine (Pfizer, Italy) were the only restraints used.

The SDFT lesion was injected by a longitudinal technique (preferred in these cases by the operator) with a 20 gauge, 70 mm needle, in a proximal-to-distal direction. The AL-DDFT lesion was injected transversely using a 21 gauge, 40 mm needle, inserted laterally. A sterile bandage was applied and maintained for 48 h. For the same period of time, the horses were box rested and then allowed out for hand walking for 15 min/day over 15 days and 30 min/day for a further 15 days. No non-steroidal anti-inflammatory drugs (NSAIDs) or antibiotics were given,

based on the aseptic technique. At this stage of the study, the follow-up period has reached just over 90 days.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad InStat 3.00 for Windows (GraphPad Software, La Jolla, CA, USA). Three replicates for each experiment (growth curve, population doublings and CFU) were performed and the results are reported as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) for multiple comparisons by Student–Newman–Keuls multiple comparison tests was used. CFU comparison among different cell plating densities inside each group (AECs or AMCs) and between groups (AECs and AMCs) of the same cell density were analysed. $p < 0.05$ was considered as significant.

3. Results

3.1. Amnion collection and isolation of amniotic epithelial and mesenchymal cells

The cellular yield from term amnion was about 10×10^6 AECs and 5×10^6 AMCs (i.e. two-fold more AECs than

AMCs). The initial viability was >75% for both cell populations.

Due to the lack of equine MSC-specific cell-surface markers, the cells were selected purely on their ability to adhere to plastic. Isolated cells readily attached to plastic culture dishes. AECs displayed typical polygonal epithelial morphology, while AMCs were fibroblast-like (Figure 1bA, bA, C). When both cell lines reached confluence, spheroid clusters appeared as three-dimensional (3D) structures in the dish. This clustering was consistently observed when cultures reached confluence at every subsequent passage (Figure 1bB, D).

After thawing (at P0), the vitality was 60% for AECs and 80% for AMCs. AECs and AMCs conserved their shapes, polygonal epithelial and fibroblast-like, respectively.

3.2. Proliferation assays

AMCs at P1 demonstrated a growth curve with an initial lag phase of 0–24 h and subsequent log phase (1–13 days) that was more intensive at P5. At P10, which was the last time point included in our study, the AMCs showed less extensive proliferation (Figure 1cA).

The AECs demonstrated a growth curve with an initial lag phase longer than that registered for AMCs (0–48 h) and subsequent log phase (2–13 days) that decreased at P5 and P10 (Figure 1cB). The AECs showed slow adhesion to culture substrates and less proliferation than AMCs.

Doubling time for AECs significantly increased ($p < 0.05$) after P6 compared to earlier passages, remaining constant until P10, while for AMCs, the proliferative ability decreased after P8 ($p < 0.05$) but was very intensive between P4 and P6, as confirmed by the growth curve at P5 (Figure 1cC). Differences between AEC and AMC doubling times were observed. AECs showed a statistically different (higher) doubling time with respect to AMCs ($p < 0.05$). Cell population doublings after 31 days were 13.06 ± 0.21 and 26.61 ± 0.15 for AECs and AMCs, respectively. At P10, even though we observed a decrease of proliferative rate, the doubling time was still low and < 2 days.

After thawing and 31 days of culture, 12.61 and 16.52 PD were observed for frozen AECs and AMCs, respectively (Figure 1dA, B).

3.3. CFU assays

The number of cell colonies formed was counted at P0 after seeding cells at different density/cm². For each cell population (AECs and AMCs), the results demonstrated a statistically significant increase in CFU frequency with increasing cell seeding densities. For each density of seeding, AMCs showed a significantly higher CFU ($p < 0.05$) in comparison to AECs (Table 2).

3.4. Multipotent differentiation

The differentiation potential of AMCs and AECs was evaluated at P3.

Table 2. CFU assay

	Density cells/cm ²	Total cells	CFU	1 CFU each
AECs	100	950	1 ± 0 ^{a,A}	950
	250	2375	10.67 ± 1.53 ^{b,A}	222.66
	500	4750	20 ± 2 ^{c,A}	237.50
	1000	9500	30 ± 2 ^{d,A}	316.67
AMCs	100	950	1.33 ± 0.58 ^{a,B}	712.50
	250	2375	20.33 ± 2.52 ^{b,B}	116.80
	500	4750	59.67 ± 4.93 ^{c,B}	79.61
	1000	9500	74.67 ± 2.52 ^{d,B}	127.23

Different small letters superscripts (a,b,c,d) indicate statistically different comparisons ($p < 0.05$) between cell densities in each group (AECs or AMCs). Different capital letter superscripts (A, B, C, D) indicate statistically different comparisons ($p < 0.05$) between the same cell density among groups (AECs and AMCs).

- *Osteogenic differentiation potential.* After 10 days of induction, osteogenic differentiation was confirmed by von Kossa staining, which was more intensive in AMCs than AECs, while the control was negative for the staining. RT-PCR analysis of *OPN* and *BGLAP* mRNA expression confirmed osteogenic induction. Amnion-derived cells showed an increased expression of *OPN* and *BGLAP* genes when compared to controls (Figure 2a).
- *Adipogenic differentiation potential.* Amnion-derived cells were able to undergo adipogenic differentiation, as demonstrated by the development of positive staining for oil red O after 3 weeks of culture in adipogenic induction medium, while cells maintained in regular control medium showed no lipid deposits. AMCs exhibited more intensive staining than AECs. RT-PCR analysis of *PPAR-γ* and *ADIPQ* mRNA expression confirmed adipogenic induction (Figure 2b).
- *Chondrogenic differentiation potential.* Chondrogenic differentiation of AMCs and AECs was identified by marked deposition of glycosaminoglycans in the matrix, which was observable after Alcian blue staining. The presence of *COL2A1* and *ACAN* mRNA confirmed chondrogenic induction for both cell populations (Figure 2c).
- *Neurogenic differentiation potential.* After 3 days of induction, neurogenic differentiation was confirmed by the morphology of the cells. The amnion-derived cells adopted a morphology typical of neural cells, with dendrite-like processes. The presence of *GFAP* mRNA suggests that under these culture conditions, amnion-derived cells were induced to differentiate into glial cells. Both cell populations lacked *NES* expression (Figure 2d).

The RT-PCR analysis of equine adult tissues (bone, fat, cartilage and spinal cord) showed expression of the specific genes studied. The frozen-thawed cells were able to differentiate toward the same lineages tested for freshly isolated cells (data not shown).

3.5. Histology and immunocytochemistry

The allanto-amnions from horse term fetal membranes were characterized by epithelial and mesenchymal layers.

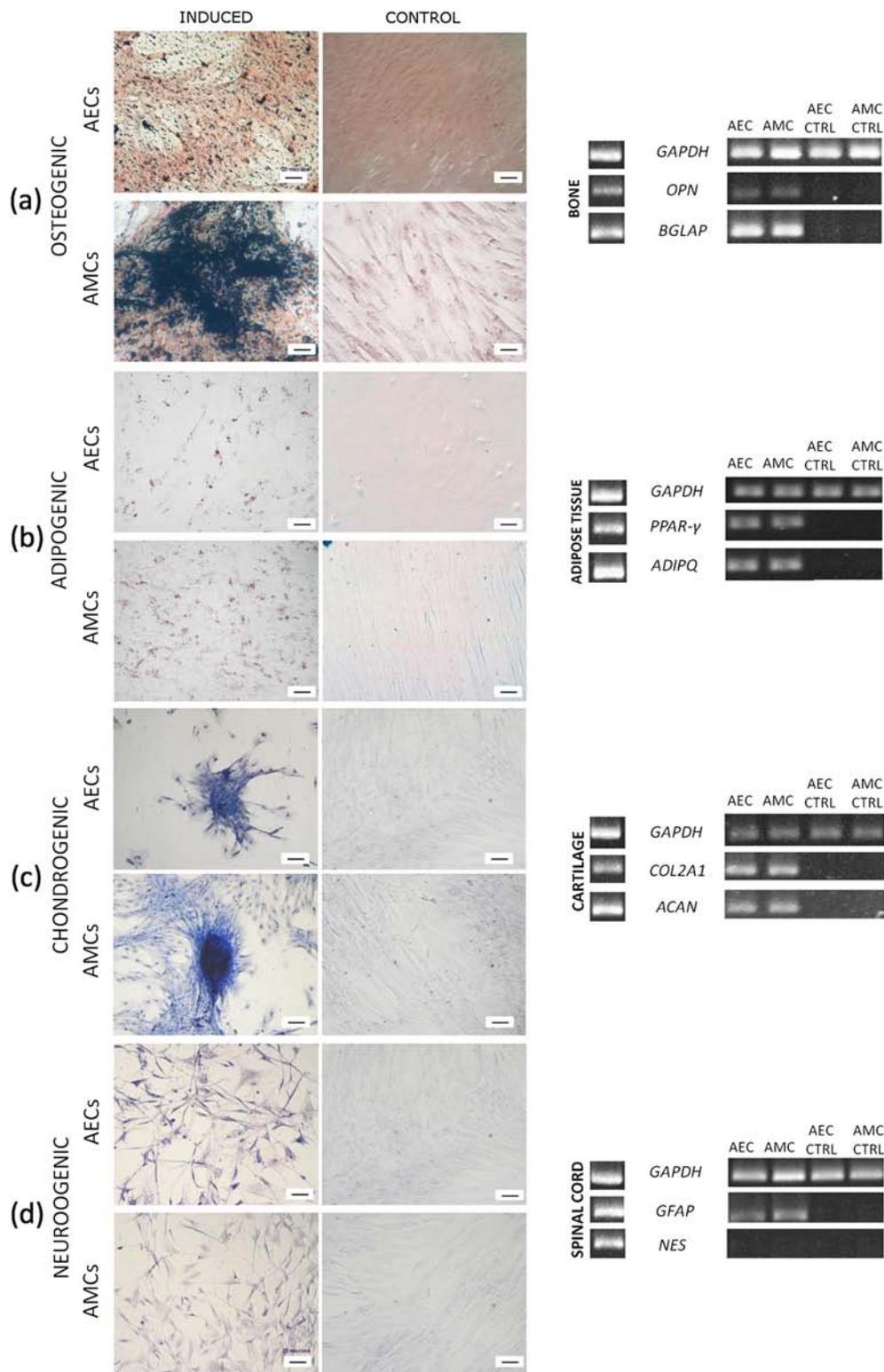


Figure 2. Staining of differentiated and control undifferentiated equine AECs and AMCs and respective molecular expression. (a) von Kossa staining after osteogenic induction and RT-PCR analysis of osteopontin (OPN) and osteocalcin (BGLAP). (b) Oil red O-stained cytoplasmic neutral lipids after adipogenic induction and RT-PCR of PPAR- γ and adiponectin. (c) Alcian blue staining after chondrogenic induction and RT-PCR of collagenase (COL2A1) and aggrecan (ACAN). (d) Nissl staining after neurogenic induction and RT-PCR of nestin and GFAP; magnification, $\times 20$; scale bar = 20 μm . GAPDH was employed as the reference gene. Bone, adipose tissue, cartilage and spinal cord were used as positive controls

Amnion is a unique avascular tissue and is comprised primarily of epithelial cells (Figure 3aA) and stromal cells (Figure 3aB). AECs create a continuous lining adjacent to the amniotic fluid on one side of the allanto-amnion. AM was digested to obtain epithelial (Figure 3bA) and mesenchymal stromal cells (Figure 3bB). Clear, strong positivity for Vimentin was detectable in the cytoplasm of the cells resident inside the mesenchymal layer of the AM (Figure 3cA) while, as expected, strong cytoplasmic PanCytokeratin positivity was revealed in the epithelial layer of AM (Figure 3cB). Vimentin was also detectable in isolated AMCs (Figure 3cC) that were predominantly PanCytokeratin-negative (Figure 3cD).

AECs were strongly positive for PanCytokeratin (Figure 3cE-F) and negative for Vimentin (Figure 3cE), even though Vimentin-positive AECs were occasionally detected.

Expression analysis of the pluripotent stem cell markers Oct-4, SSEA-4 and TRA-1-60 was performed. AECs and AMCs expressed all three of these antigens (Figure 3d). Oct-4 was expressed mainly in the nucleus and weakly in the cytoplasm, while SSEA-4 and TRA-1-60 were expressed on the cell surface. Expression of TRA-1-60 was very weak. The adult fibroblasts used as control resulted negative (see Supporting information, File S1).

By using alkaline phosphatase (AP) staining, we observed AP-positive reactivity in cultures of both AECs

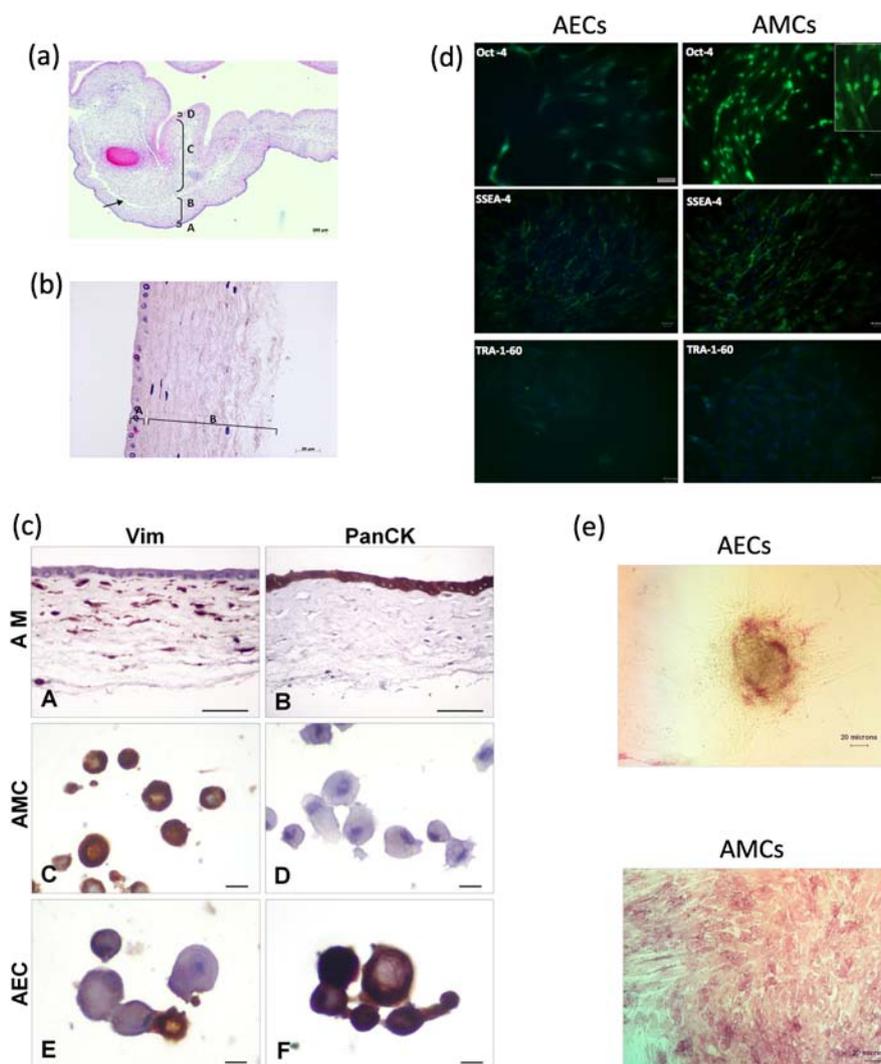


Figure 3. (a) Morphology of equine allanto-amniotic membrane at term gestation (hematoxylin and eosin staining). A: amniotic epithelium layer; B: amniotic mesenchymal layer; C: allantoic mesenchymal layer; D: allantoic epithelium layer. Contact region between amnion and allantois is showed by arrow. Scale bar = 200 μ m. (b) Histochemical section of amniotic membrane with epithelial region (A) and mesenchymal region (B). Hematoxylin and eosin staining. Scale bar = 20 μ m. (c) Immunolabelling for Vimentin (Vim) and Cytokeratins (PanCK) performed on horse amniotic membrane (AM) and derived cells (AMCs = amniotic mesenchymal cells; AECs = amniotic epithelial cells). Vimentin positive mesenchymal cells (A) and PanCytokeratin positive epithelial cell layer (B) in the context of AM. Immunorexpression of Vimentin and PanCytokeratin in AMCs (C and D respectively) and AECs (E and F respectively). Indirect immunoperoxidase, DAB staining. Photomicrographs A and B, scale bar = 50 μ m. Photomicrographs C to F, scale bar = 10 μ m. (d) Photomicrographs of immunostaining of AECs and AMCs. Cells labelled with antibodies against antigens Oct-4, SSEA-4, TRA-1-60. Magnification 20x, scale bar = 20 μ m. (e) Positive reaction to alkaline phosphatase of spheroid structures in AECs (A) and of monolayer of AMCs (B)

and AMCs (Figure 3eA, B). Furthermore, AP-positive spheroid structures were observed (Figure 3eA). After thawing, AECs and AMCs still showed positive immunostaining for Oct-4, SSEA-4 and TRA-1-60 (data not shown).

3.6. RNA extraction and RT-PCR analysis

To characterize amnion-derived cells, we performed RT-PCR on total RNA isolated from cells at different passages. At the transcriptional level, both cell populations expressed MSC markers (*CD29*, *CD44*, *CD166* and *CD105*) at P1 and P5. In both cell populations, expression of the *CD34* marker was not registered at P1 but was present at P5. *CD34* expression was also investigated at other passages (P2, P3, P4, P7 and P10). This haematopoietic marker was found to be stably expressed, starting from P5 until P10. Both AECs and AMCs expressed *MHC-I* at P1 and P5 and lacked *MHC-II* expression at P1, but began to express this marker at P5 (Figure 4a).

After thawing, cells studied at P3 expressed the same MSC-mRNA markers of freshly isolated cells, such as *CD29*, *CD44* and *MHC-I*, but not *CD34* and *MHC-II*. Frozen samples showed decreased *CD105* gene expression with respect to freshly isolated cells. Only AECs were positive for *CD166* (Figure 4b).

3.7. Cell transplantation *in vivo*

To investigate their ability to exert beneficial effects *in vivo*, horse AMCs were allogeneically transplanted into three horses with tendon lesions. Specifically, the first horse (case 1) was affected by an acute rupture of a forelimb superficial digital flexor tendon (SDFT), involving 80% of the tendon cross-section (Figure 5a, b, case 1). In the second horse (case 2), an obvious severe SDFT core lesion of 0.58 cm² was detected in a forelimb (Figure 5a, b, case 2). Case 3 was a horse with an acute lesion of a forelimb accessory ligament of the deep digital flexor tendon (AL-DDFT) and the damaged area was quantified to 2.32 cm² (Figure 5a, b, case 3).

When horse AMCs were allogeneically transplanted into these horses, all surgical procedures were clearly well-tolerated. No soreness or flares were noticed as a result of the surgery. Our patients were able to walk and turn sharply soon after the implant, especially considering that no NSAIDs were administered. An early reduction in the degree of lameness, along with an appreciable volume reduction and decreased soreness on palpation, occurred in the treated horses just a few days after cell implantation.

In case 1, the anechoic wide area, representative of the lesion, was markedly improved. Ecogenic appearance and tissue architecture in a longitudinal scan revealed a clear healing process over a period of 70 days (Figure 5c, d, case 1). In case 2, the anechoic core lesion, which measured 0.58 cm², was reduced to an ipoechoic 0.18 cm² area together with a satisfying longitudinal ultrasound pattern of the tendon fibres in nearly 60 days (Figure 5c, d, case 2). In case 3, the severe 2.32 cm² acute lesion in the AL-DDFT became non-ecographically detectable within 60 days (Figure 5c, d, case 3). Furthermore, follow-up examinations were carried out in all cases during retraining and at 6 months after cell treatment, with no failures observed.

4. Discussion

In the present study, for the first time, AM isolated from horse term placenta, which is easily accessible and usually discarded after birth, has been evaluated as an alternative source for the isolation of progenitor cells. Following enzymatic digestion of the amniotic tissue, adherent equine amnion-derived cells were expanded in culture until sufficient numbers were available for characterization, differentiation and allogeneic grafting studies.

The first set of data resulting from our *in vitro* differentiation studies suggests that these cells have high plasticity, as supported by their capacity to differentiate into multiple germ layers (mesoderm and ectoderm). In particular, as already reported by In 't Anker *et al.* (2004) for amniotic cells isolated from human placenta, here we have shown that horse amnion-derived cells are also able to differentiate toward the osteogenic, adipogenic and chondrogenic lineages. We have also demonstrated that

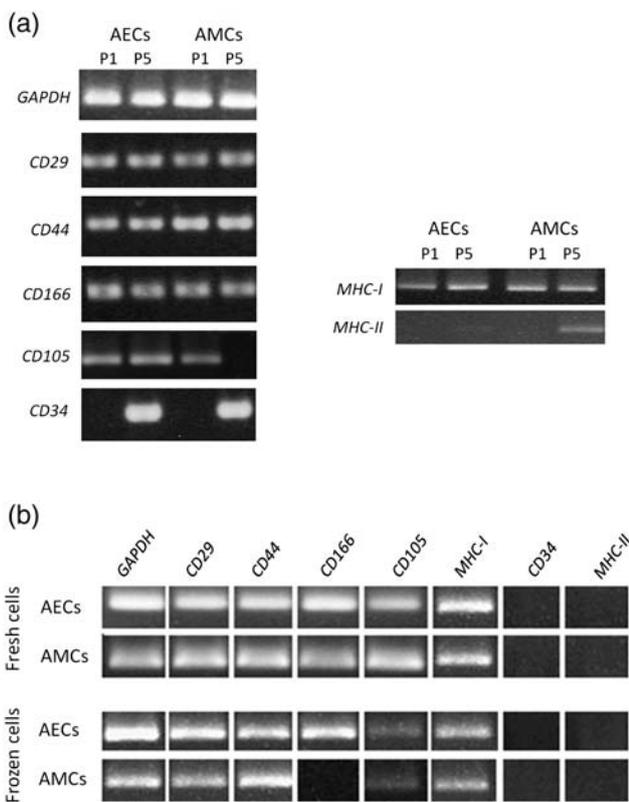
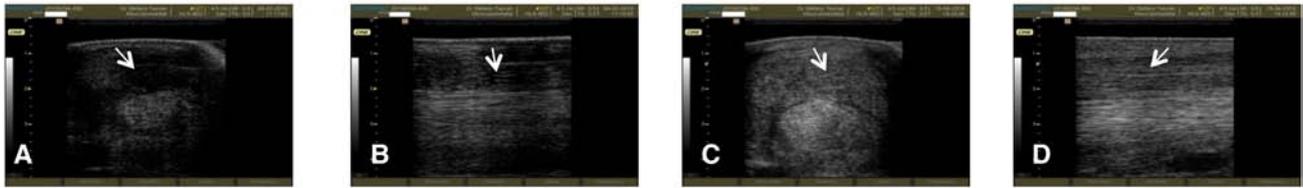
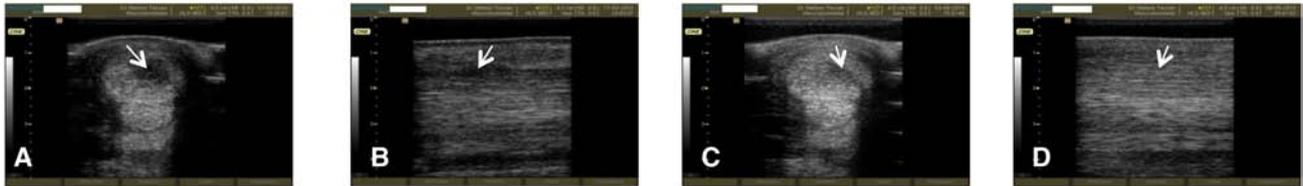


Figure 4. (a) RT-PCR analysis of mesenchymal (*CD29*, *CD44*, *CD166* and *CD105*), haematopoietic (*CD34*) specific gene expression on AECs and AMCs at P1 and P5. Major histocompatibility complex (MHC) I and II gene expression is also reported. (b) Comparison of gene expression between fresh and frozen cells. *GAPDH* was used as the reference gene

Case 1



Case 2



Case 3

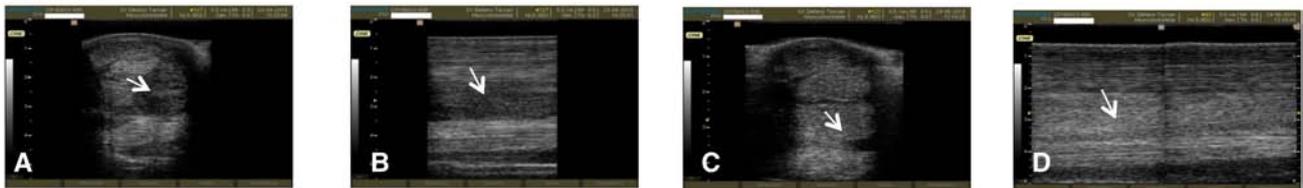


Figure 5. (Case 1) Acute rupture of a forelimb SDFT. Arrows show anechoic wide area in transverse (A) and longitudinal (B) ultrasound scans. Arrows in (C, D) show homogeneously echogenic area in transverse and longitudinal ultrasound sections, respectively, in the same lesion 50 days after cells implantation. (Case 2) Severe SDFT core lesion in a forelimb SDFT. Arrows show anechoic area in transverse (A) and longitudinal (B) ultrasound scans, and slightly ipoechoic area in transverse (C) and longitudinal (D) ultrasound sections, respectively, in the same lesion 50 days after implant. (Case 3) Acute lesion of a forelimb AL-DDFT. Arrows show ipoechoic area in transverse (A) and longitudinal (B) ultrasound scans and echogenic area in transverse (C) and longitudinal (D) ultrasound sections taken 50 days after cells implantation

horse amnion-derived cells are able to differentiate toward the glial cell lineage, as previously shown by Miki *et al.* (2005) for cells isolated from human amnion, where 95% of amniotic epithelial cells showed immunolocalization of GFAP.

Due to their ability to differentiate toward multiple cell types, equine amnion-derived cells could play a key role in the development of cell-based strategies as a potential alternative to tissue/organ transplantation.

Regenerative medicine is an emerging and multidisciplinary field which draws on biology, medicine and genetic manipulation for the development of strategies aimed at maintaining, enhancing or restoring the function of tissues or organs which have been compromised by disease or injury. In veterinary medicine, AM *in toto* has already been used in clinical therapy. The present study is the first to document the use of equine amnion-derived cells to treat tendon injury in horses. The beneficial effects of these AM-derived cells in these initial tests underline their potential application in veterinary regenerative medicine.

Through cell transplantation studies *in vivo*, we found that the transplanted equine amnion-derived cells were well tolerated by horses, and all of the clinical findings reported (i.e. the quick reduction in gross tendon size, palpation sensitivity and ultrasonographic cross-sectional area measurements), provide compelling evidence to support the exertion of beneficial effects by the injected cells. The ultrasonographic evolution reported for

tendon and ligament architecture are similar to what has been previously reported after application of other cultured autologous mesenchymal multipotent cells (for a review, see Richardson *et al.*, 2007). Interestingly, even though our study included only a limited number of cases, we did not observe any relapse of the tendon defect, as often observed after using BM-MSCs (S. Tassan, personal communication, 2010; see Supporting information, File S2) or other treatment, such as platelet rich plasma (PRP) (Rindermann *et al.*, 2010). The possibility of administering an immediate intralesional treatment which is available before any ultrastructural change is observed within the injured tendon, together with the plasticity effect of AMSCs, represent the major features of interest for this novel biotechnological approach to equine tendinopathies. In this study we intended to demonstrate the feasibility of using amniotic-derived cells in horse tendon injuries without making comparison with other cell types, which would have needed many more spontaneously occurring clinical cases or the use of experimental horses. This study was not a preclinical trial but was, rather, an analysis of the preliminary findings obtained with these equine amnion-derived cells. Obviously, a weak point of this study is the lack of a control group and of histological evaluation. Future studies, applied to a greater number of horses, are needed to confirm these results. Given that this is the first report on the isolation of equine amnion-derived cells and their potential clinical use, we have performed a detailed study

regarding the morphology of horse AM and the characterization of its derived cells. Based on our histological findings, horse AM is composed of an epithelial layer and an avascular stroma in which a network of dispersed fibroblast-like mesenchymal cells are embedded. These cells were shown to be cytokeratin- and vimentin-positive, confirming their epithelial or mesenchymal nature, respectively. A few cells in both cell lines were positive for the marker normally associated with the other lineage. These rare positive reactions to these non-specific lineage markers could indicate that *in vitro* culture or environmental influences may induce dedifferentiation of these cells (Miki and Strom, 2006). Alternatively, this finding could be ascribed to the heterogeneity of the cell populations that were obtained after enzymatic digestion.

Under our experimental conditions, after digestion, the initial cellular vitality was >75%, and this is a good parameter of the long-term quality of the cells in view of their plating efficiency and cell growth. The proliferation assay demonstrated that epithelial cells had lower plating efficiency than mesenchymal cells, due to the initial lag phase of 48 and 24 h, respectively. Nonetheless, horse amnion-derived cells demonstrated a high proliferative capacity until passages 6–8. After this time, proliferation decreased in both cell lines, but AMCs remained superior to AECs in terms of proliferative ability in terms of both total cell number and doubling time. We showed a decrease in cell proliferation at the higher passages but at P10, the last time point considered in our study, we could still demonstrate a DT value of <2 days, which is lower than the mean DT value of equine BM-MSCs (which is ca. 3.35 days; data not shown; see Supporting information, File S3). However, robust proliferation until P6 is also reported for human amniotic cells by other authors (Soncini *et al.*, 2007; Miki *et al.*, 2010). From our results, when 5×10^5 cells are seeded, 8×10^6 amnion-derived cells are available in 5 days, while 14 days are needed using BM-derived cells. Fortier and Smith (2008) reported that in the equine species a 2–3 week culture period is needed to expand the selected cells from BM until $> 10 \times 10^6$ cells are available for implantation into the tendon core lesion, representing a disadvantage for the delay between BM aspiration and implantation. Also in human medicine, a lower doubling time is a common characteristic of the MSCs isolated from the umbilical cord matrix and cord blood compared to MSCs isolated from bone marrow (Campagnoli *et al.*, 2001; Baksh *et al.*, 2007; Karahuseyinoglu *et al.*, 2007; Lund *et al.*, 2007). This behaviour could reflect the more primitive nature of fetal adnexa when compared to cells isolated from bone marrow (Weiss *et al.*, 2007).

Equine AECs and AMCs kept in high-density culture developed spheroid structures without showing cell contact-inhibition, as previously reported for human AECs (Miki *et al.*, 2005). Amnion-derived cells continued to grow by cell multilayering and formed aggregates overlying a layer of confluent cells, as previously described also by Carlin *et al.* (2006) for pig umbilical cord matrix-derived cells. The high level of alkaline phosphatase staining could suggest the undifferentiated state of horse amnion-derived cells.

The cell monolayers also had the ability to give rise *in vitro* to clones with frequency that increased with cell-seeding densities up to 1000 cells/cm², indicating some paracrine signalling between amniotic cells, which may potentiate CFU formation in primary culture (P0) (Sarugaser *et al.*, 2005).

Amnion-derived cells displayed gene expression for the common and well-defined MSC markers *CD29*, *CD44*, *CD166*, *CD105* and *MHC-I*, while they were negative for *MHC-II*. The amnion-derived cells at P0 lacked *CD34* expression, although this marker started to be expressed at P5 in both cell populations (AMCs and AECs). This could constitute evidence that amniotic cells have an angiogenic potential (Alviano *et al.*, 2007). At P5, *MHC-II* also started to be expressed, while *CD105* expression was lost. Taking this into consideration, amniotic-derived cells should probably be used only after a few culture passages. It is known that to heal large defects, a huge number of cells would be required, but this would not represent a problem, especially in the horse because, the amnion being a large membrane, a required huge number of cells could be easily obtained, starting simply from a bigger portion of the membrane. This represents an advantage compared to other cell sources (BM, fat, blood umbilical cord), which are available only in limited amounts. Anyway, the poor stability of the expression of this marker may indicate that epigenetic phenomena associated with cell culture could affect the amnion-derived cells. Further characterization of the amnion-derived cells is needed to understand the mechanisms underlying these changes.

In addition to characteristic MSC markers, horse amnion-derived cells showed immunopositivity to Oct-4, TRA-1-60 and SSEA-4, which represents the pattern previously reported for pluripotent stem cells (Portmann-Lanz *et al.*, 2006). One interpretation of these findings is that amnion-derived cells are pleiotropic and express a relatively large number of genes in relatively low abundance (Weiss *et al.*, 2006). On the other hand, it may serve as evidence that the amnion-derived cell populations have a subset of primitive stem cells. In this regard, it is noteworthy that the epithelial layer of horse amnion originates from the trophoblast and is continuous with the epiblast (Vejlsted, 2010). It is therefore reasonable to speculate that some AECs may have escaped the specification that accompanies gastrulation, and that these cells may retain some or all of the characteristics of epiblastic cells, such as pluripotency (Miki and Strom, 2006). Regarding Oct-4, usually localized in the nucleus, the weak immunocytochemical positivity in the cytoplasm observed in our experiment could very likely be associated to the problem of antibody specificity in the horse. Unfortunately, no horse-specific antibodies are available, then there is possibly a less specific localization of the antigen protein inside the amnion cell studied.

From our results, both AECs and AMCs exhibit specific MSC-like properties, with no apparent difference between the two lineages, except for proliferative capacity and intensity of lineage-specific staining after differentiation

toward the mesodermal lineages, which was not detectable by molecular analysis.

This study is the first to document the use of amnion-derived cells in horses with ligament injuries, demonstrating that these cells were well-tolerated after allogeneic treatment. Moreover, these results led us to develop protocols for the isolation of horse amnion-derived cells that could be used in the future for veterinary regenerative therapies. Therefore, once technology is established to isolate and store equine amnion-derived cells, it should be possible to create an equine amnion cell bank which could be used for allogeneic clinical applications. To this end, we have shown that equine amnion-isolated cells can be frozen, stored and recovered without loss of their functional integrity in terms of morphology, presence of specific stemness markers and differentiation potential, although renewal capacity was lower than that observed in freshly isolated cells. In this view, cryopreservation protocols should be improved in order to allow creation of a cell banking service.

5. Conclusions

Our preliminary data show that equine amnion holds evident promise as a source of presumptive progenitor cells which may have widespread clinical applications. Amnion-derived cells can be isolated from a non-controversial, plentiful source, which can also be harvested non-invasively and at low cost. Moreover, these cells are not acutely rejected when transplanted to treat tendon injuries.

Therefore, although further studies are required to fully understand the immunological properties and the potential of amnion-derived cells as valuable candidates for cell therapy strategies in various preclinical experiments, it is highly likely that these cells may have widespread clinical applications in veterinary and regenerative medicine.

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Supporting information on the internet

The following supporting information may be found in the online version of this article.

File S1. Negative control of horse skin fibroblasts, Oct-4

File S2. Comparison between BM-MSCS or amnion-derived cells treatment of injured tendons in horses

File S3. Comparative analysis of proliferative characteristics between amnion-derived cells and bone marrow MSCs

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