Stem Cell Research 37 (2019) 101442

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Analysis of mesenchymal cells (MSCs) from bone marrow, synovial fluid and mesenteric, neck and tail adipose tissue sources from equines

Magdalena Arévalo-Turrubiarte, Chiara Olmeo, Paolo Accornero, Mario Baratta, Eugenio Martignani*

Department of Veterinary Science, University of Turin, Largo Braccini 2, 10095 Grugliasco, TO, Italy

ARTICLE INFO

Keywords:
Mesenchymal stem cells
Equine
Proliferation
Differentiation

ABSTRACT

Mesenchymal stem cells (MSCs) have been used in equines as an alternative therapy. A comparative study about the phenotype and *in vitro* performance of different MSCs tissue sources in adult equines was needed. This study might serve to provide the knowledge to select a valuable harvesting source of MSCs. Bone marrow, synovial and adipose (mesenteric, neck and tail fat) tissues were collected from adult equines.

Cell surface markers expression (CD11α/CD18, CD45, CD79α, CD90, CD105 and MHC II) and *in vitro* differentiation assays were made. *In vitro* cell migration, cell growth and wound healing capacity tests helped to study their behavior and properties. MSCs phenotype was positively confirmed by the cell surfaces markers and a tri-lineage differentiation profile. Bone marrow cells showed the highest migration capacity, while synovial fluid cells displayed the highest cell growth. Bone marrow cells showed a better wound healing when compared with all the different MSCs. We conclude that bone marrow, synovial and adipose tissue derived from adult equines are a good source for cell therapy but they conserve different functional properties: bone marrow showed an interesting migration and wound healing capacity while synovial fluid cells and their highest cell growth suggest that these MSCs would yield higher cell numbers in a shorter time.

1. Introduction

Depending on their physical activity, equines' joints are exposed to injuries having a negative impact on articulation tissue integrity and homeostasis (Schlueter and Orth, 2004). During high locomotion activities, *i.e.* horse racing, there is a constant stress on leg joint structure to bear weight (Schlueter and Orth, 2004). This might cause trauma that later would develop in diseases like osteoarthritis. Other factors that predispose to osteoarthritis are the incorrect leg conformation and progressive damage degeneration in the joint of elderly equines (Schlueter and Orth, 2004). In equine medicine, different therapies like the debridement of tissue, osteochondral implantation and grafting techniques aim to improve joint problems (Ortved and Nixon, 2016). Cell implantation of autologous or allogeneic cells in the injured tissue seems to be a useful therapy showing tissue healing and a reduction in size of the injured sites, thus appearing as a less invasive therapy (Hipp and Atala, 2008). After cell implantation, an improvement of the histological organization of damaged tissue and a decrease of the levels of pathological pathways have been observed (Ortved and Nixon, 2016). Mesenchymal stem cells (MSCs) have been used as a source for cell implantation to repair joint damage (Hipp and Atala, 2008; Ortved and Nixon, 2016). Among MSCs sources, bone marrow (BM) cells have been largely used for implantation (Friedenstein et al., 1966). BM cells usually are harvested from sternum or the ilium aspirates (Lombana et al., 2015). The BM source is composed by a heterogeneous cell population: mesenchymal fibroblast-like cells, hematopoietic cells and diverse cell population that contribute to their differentiation capacity *in vitro* (Elsafadi et al., 2016; Friedenstein et al., 1966). BM cells can be obtained from different anatomical regions in the equines. Differences between cell sources *i.e.* from ilium and sternum regions seem to be minimal in respect to their multi-lineage differentiation (adipogenic, osteogenic and chondrogenic) capacity on cells obtained from young equines (Lombana et al., 2015). Other MSCs sources than BM have been evaluated to be used in regenerative medicine. In 2002, Zuk et al. (Zuk et al., 2002) showed adipose cells differentiation into mesodermal cells demonstrating their differentiation capacity into other cell types. Adipose cells have also been used to be differentiated into other cell types showing a higher proliferative ability and doubling time in comparison with BM cells (Colleoni et al., 2009). One of the advantages of choosing adipose tissue as a source when obtaining MSCs is the higher amount of

* Corresponding author.
E-mail address: eugenio.martignani@unito.it (E. Martignani).

https://doi.org/10.1016/j.scr.2019.101442
Received 6 November 2018; Received in revised form 19 March 2019; Accepted 15 April 2019
Available online 17 April 2019

cells obtained after tissue digestion (Metcalf et al., 2016). However, the region of adipose sampling yields differences in the number of viable cells. Cells obtained from mesenteric adipose tissue show a higher viability and more nucleated cells than those from tail head adipose tissue in equines (Metcalf et al., 2016). In respect with the frequency of stem cell number it seems they are more abundant on the abdominal adipose tissues than in other adipose regions (Jurgens et al., 2008). The expression of cell surface markers has helped to study the phenotype of these cells (De Schauwer et al., 2011). The expression of CD90 cell surface marker related with stemness and adipogenesis (Woeller et al., 2015) and the expression of CD105 have been reported to be higher in the omental fat than in the subcutaneal adipose tissue in dogs (Requicha et al., 2012). In addition to adipose cells, synovial fluid cells have also been suggested as an important source of MSCs. Synovial fluid cells have been suggested to play an important role on cartilage regeneration in equines (Murata et al., 2014). Apparently, synovial fluid cells possess a high chondrogenic potential when compared with adipose and BM cells. Although synovial fluid cells isolated from healthy equines do not seem to be more abundant when compared with diseased joints, synovial fluid remains an important source to obtain MSCs (Murata et al., 2014). As mentioned before, synovial fluid seems a good alternative MSCs harvesting source also because joints are a more easily accesible and less invasive site by arthrocentesis.

Previous studies have shown that the anatomical sampling site remains decisive for MSCs yield and might influence cell inner characteristics (Metcalf et al., 2016; Requicha et al., 2012). Moreover the overall donor horse condition has a strong impact on isolated MSCs. For instance, adipose tissue cells (subcutaneous tail fat) obtained from horses presenting equine metabolic syndrome represent an inefficient MSCs source due to a decrease in cell proliferation rate, a less effective osteogenic and chondrogenic differentiation, mitochondrial alteration and an increased autophagy in cells (Marycz et al., 2016a; Marycz et al., 2016b). This metabolic disorder is predisposed in ponies, Andalusian and other equine breeds, it's influenced by the type of diet and related to laminitis, an inflammatory disease in the hoof which in turn is related with osteoarthritis (Bamford et al., 2014). In addition, stemness, cell differentiation capacity and cell growth properties might be crucial for cell performance (Marycz et al., 2016a; Marycz et al., 2016b) within the implanted tissue and their subsequent clinical outcome. Furthermore, cell deterioration related to senescence decreases MCSs proliferation, modifications of the deoxyribonucleic acid and there is presence of high levels of oxidative stress factors (Kornicka et al., 2017). Middle-aged horses (aged 10–13 years) differ in MSCs yield and cell differentiation (Carter-Arnold et al., 2014), therefore donor conditions and MSCs differences due to horse breed, nutrition and activity should be carefully considered for each clinical case. To our knowledge, different studies (Carter-Arnold et al., 2014; Colleoni et al., 2009; Lombana et al., 2015; Metcalf et al., 2016; Murata et al., 2014; Paebst et al., 2014; Prado et al., 2015; Radcliffe et al., 2010; Ranera et al., 2011; Zayed et al., 2017) have evaluated cell sources from BM, adipose, and synovial fluid tissues, but no direct comparison of all reported sources has been carried out from the same horses to date. In most cases different tissues were sampled from different animals. In addition, a detailed evaluation about cell phenotype and cell *in vitro* performance associated with their harvesting site in adult equines has not been performed. That is, MSCs harvested from donor-matched different tissue sources (bone marrow, three different adipose tissue sites and synovial fluid) has not been reported yet, and more importantly *in vitro* assays to evaluate properties and compare behavior among donor-matched sources that are related to their potential therapeutic application were not performed on isolated cells. Thus, particular interest on different tissue MSCs sources might help clinicians about the available MSCs harvesting areas in cases where one tissue source would not be suitable due to equine's condition (Bamford et al., 2014; Carter-Arnold et al., 2014; Marycz et al., 2016b). In addition, the use of allogeneic MSCs has been proposed as an alternative therapeutic source in equines (Ardanaz et al., 2016). Therefore, the aim of this study was to evaluate cell phenotype by analyzing the expression of cell surface markers that were described for human MSCs and those reported in equines (Dominici et al., 2006; Paebst et al., 2014) and then to test functional properties of equine MSCs to evaluate cell migration, growth and wound healing capacities. This study might help in the selection of an appropriate tissue source to obtain MSCs for clinical applications in equines.

2. Materials and methods

2.1. Tissue dissociation and equine mesenchymal cells culture

Samples from three mixed breed horses (10–16 months old) were collected at the slaughter house according to the Italian regulations under the supervision of the Veterinary Services of the Italian National Health Service, branch of the Ministry of Health. Tissue samples were obtained as follows: BM (through scraping of the BM area of the sternum), synovial fluid (radio carpal knee joint) and adipose tissue from mesenteric, neck and tail base fat regions. Tissue samples were collected within one hour from the slaughtering and cell isolation began approximately one hour after collection. All sample tissues except from synovial fluid were rinsed twice with PBS before the subsequent digestion step. BM tissue (200 mg) was washed with PBS and centrifuged at 1200*g* then filtered through a 40-μm strainer. Later, BM cells were cultured in proliferation medium (high glucose Dulbecco's Modified Eagle's Medium (DMEM) 1X (Sigma-Aldrich), 10% fetal bovine serum (FBS) (Gibco), 4.5 g/l D-Glucose, 2 mM L-Glutamine, 2.5 μg/ml amphotericin B and 1% penicillin-streptomycin (all from Sigma-Aldrich). Tissue from different adipose areas (neck fat 2 g, tail fat 1 g, mesenteric fat 500 mg - approximately) were washed with PBS and cut in small pieces and digested with 250 U/ml collagenase type I (BD Bioscience) at 37 °C for 3–4 h. Then, adipose tissue was centrifuged at 1200*g*, filtered with a 40-μm strainer and cells were cultured in proliferation medium. 2 ml out of the recovered 5–7 ml of synovial fluid were gently mixed with 3 ml proliferation medium and directly seeded on culture dishes. The cells obtained from BM, synovial fluid and adipose tissue were cultured respectively in a 100 mm diameter tissue culture dish (Sarstedt). After 24 h in culture, medium was replaced with fresh one to eliminate non-adherent cells. Cells were cultured at 37 °C with 5% CO_2 in proliferation medium until confluence then split in new cell culture dishes.

2.2. Flow cytometry of equine mesenchymal cells

Cells were detached with 0.25% trypsin–EDTA (Sigma-Aldrich) then counted, washed with PBS and centrifuged at 500*g* for 5 min. Aliquots of 250,000 cells per tube were prepared and washed with PBS. Cells were then incubated at 4 °C in the dark with CD11α/18-FITC (clone CVS9 horse, 1:160) (BioRad), CD45-Alexa Fluor 488 (clone F10-89-4 human, 1:160) (BioRad), CD79α-Alexa Fluor 488 (clone HM57 human, 1:80) (BioRad), CD90-PE (clone Thy1-A1human, 1:400) (R&D Systems), CD105 (clone SN6 human 1:80) (BioRad) and MHC-II-FITC (clone CVS20 horse, 1:80) (BioRad) antibodies for 15 min. The cells labelled with the CD105 antibody were then washed and incubated with an anti-mouse IgG Pacific blue-conjugated secondary antibody (1500) (Invitrogen). Cells were washed with PBS and tubes were centrifuged at 500*g* for 5 min at 4 °C. Cells were re-suspended with PBS for further flow cytometry analysis. Control samples served to establish acquisition parameters on each cell type to select the population of interest. For data analysis 20,000 events were collected using an Attune® Cytometer (Life Technologies) equipped with a 405 nm and a 488 nm laser. The data obtained was analyzed with the Attune® Cytometric Software.

2.3. Cell tri-lineage differentiation

To evaluate equine mesenchymal cell adipogenic differentiation capacity, 5000 cells/well were plated in a 6-well dish (CytoOne, Starlab). The osteogenic differentiation capacity of cells was evaluated by seeding 15,000 cells/well in a 6-well dish (CytoOne, Starlab). For the chondrogenic differentiation, 5×10^5 cells were seeded in a 2 ml tube with round bottom to allow a three-dimensional spontaneous cell aggregation. Differentiation StemPro® (Gibco) medium was used for adipogenic, osteogenic and chondrogenic differentiation respectively according to manufacturer instructions. For all cell differentiation cultures, medium was replaced every two days and cells remained in culture for around 14-days in the case of adipogenesis and chondrogenesis, while for osteogenic differentiation, two stages were tested: positivity to alkaline phosphatase activity (~7 days of culture) and up to 21 days for final osteogenic differentiation.

2.4. Histological evaluation of cell tri-lineage differentiation

After culturing the cells in the appropriate differentiation medium, the medium was removed and cells were washed with PBS carefully. Cells cultured in adipogenic medium were fixed with 4% formalin and subsequently stained with Oil Red O. Pre-osteogenic differentiation was analyzed with the alkaline phosphatase staining kit (Sigma-Aldrich) following manufacturer's instructions. For osteogenic differentiation cells were fixed with 4% of formalin and stained in the dark with Alizarin red. Chondrogenic cells formed a micromass structure which was carefully washed with PBS and fixed overnight with 4% formalin. The micromass structures were incubated in 80% ethanol and then embedded in paraffin. Sections were cut at a 5 μm thickness. The slides were deparaffinized and incubated in Safranin O. Slides were mounted with EUKITT® mounting medium and images were taken after 24 h.

2.5. Cell migration

Three replicate wells were set up for each different cell type (BM, synovial fluid and adipose tissues) from each of the three horses. A total of 50,000 cells/well were plated on 8 μm TC-inserts (Sarstedt) in 24-dish (CytoOne, Starlab) with proliferation medium. After 24 h of incubation, medium was removed, then inserts were washed with PBS and the upper side of the membrane was cleaned carefully with a cotton swab. Inserts were fixed with 4% formalin for 20 min, after fixation they were washed with deionized water and the upper side of the membrane cleaned again with a cotton swab. Finally, inserts were incubated with DAPI and analyzed with an inverted fluorescent microscope (Leica as previously described). An image of the entire lower side of the TC-insert membrane was captured with a 10x objective using the LAS AF software and nuclei were counted with ImageJ software.

2.6. Mesenchymal equine cells growth assessment

To estimate cell growth, each cell type was seeded in a 6-well plate (CytoOne, Starlab) with proliferation medium at a density of 20,000 cells/well. The experiment was performed from each of the three horse's harvested tissue, and duplicates were made on each different type of tissue. Cells were incubated and cell numbers were assessed at 0, 24 and 48 h. The medium was discarded and cells were fixed with methanol-acetone (1:1 vol/vol) which was subsequently replaced with Tris-HCl buffered saline (0.1 M Tris-HCL, 8 g/l NaCl, pH 7.6). Cells were incubated with 4′6-diaminide-2′-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) to stain nuclei and images were acquired at 10x magnification in five different random areas per well with an inverted fluorescent microscope Leica AF6000 LX equipped with a Leica DFC350FX digital camera (Leica Microsystem, Wetlar, Germany). Images were analyzed with ImageJ software (1.48version) to automatically count the stained nuclei in each image. Cell growth was expressed as ratio of nuclei counted at 24 h or 48 h and the nuclei count from the same well at time 0.

2.7. Wound healing assay

The wound healing assay (or scratch assay) consisted on creating a gap in a confluent cell monolayer, capturing the images to quantify the gap filling rate. Endothelial cells harvested from equine carotid artery were kindly provided by Dr. Walid Azab from the Free University of Berlin (Freie Universität Berlin, Institut für Virologie Robert von Ostertag-Haus-Zentrum für Infektionsmedizin, Germany). Endothelial cells were seeded at a density of 150,000 per well in collagen coated 24 well plates (CytoOne, Starlab) and cultured with DMEM supplemented with 20% FBS and 1% non-essential amino acids (aa) until confluence (usually 24 h). When cells reached confluence, a scratch was made with a 1000 μl tip. Cell monolayers were washed with PBS and the medium was replaced with one suitable for the appropriate experimental condition. Briefly, two conditions were set up and for each one 3 replicate wells were prepared: co-culture with MSCs or MSCs conditioned medium. In the co-culture condition 24,000 MSCs at passage 1 from each tissue source were seeded in individual transwells (Sarstedt TC-inserts with a 1 μm porous membrane). Culture medium was switched to DMEM 2% FBS with 1% non-essential aa. As control, transwells prepared with 24,000 endothelial cells were used and processed in the same way. To test the effect of conditioned medium, MSCs were seeded at passage 0 until confluence. Culture medium was then changed to fresh DMEM 10% FBS. After 48 h conditioned medium was collected and immediately added to the appropriate wells were the scratches on endothelial cells were already performed. For this condition control was set up using 48 h conditioned medium from endothelial cells (DMEM 10% FBS). Images were acquired with a 10x objective at the time the scratches were performed (time 0) and after 16 h in culture. Three adjacent areas were photographed per each well. The images were then analyzed by Image J software with the MRI Wound Healing Tool to calculate gap areas. Gap areas at 16 h were normalized to their respective time 0 measurement and therefore data are presented as a ratio.

2.8. Statistical analysis

Flow cytometry data are expressed in percentages as mean ± standard deviation. For cell growth and cell migration results normalization was made and differences among the different tissue sources were estimated by a non-parametric test Kruskal-Wallis followed by a *post-hoc* pairwise comparison Wilcoxon-Mann-Whitney test. Wound healing statistical analysis was performed by a Kruskal-Wallis independent sample test followed by a *post-hoc* pairwise comparison Wilcoxon-Mann-Whitney test. A p-value of $< .05$ was considered statistically significant. The statistical analysis was performed with SPSS Statistics version 24 (IBM SPSS Statistics®, USA).

3. Results

After tissue dissociation, cells were cultured *in vitro* with proliferation medium (Supplementary Fig. 1) until confluence. An important characteristic of MSCs is to adhere to plastic after culture. Subsequently, cells were analyzed by flow cytometry with cell surfaces markers that have been described in mesenchymal cells of several species, including the equine one (De Schauwer et al., 2011; Dominici et al., 2006).

3.1. Flow cytometry

BM, synovial fluid and adipose tissues are constituted by different type of cells. To identify mesenchymal cells, cultured cells were analyzed by flow cytometry using cell surface markers CD11α/CD18,

CD45, CD79α, CD90, CD105 and MHC Class II. We show representative plots of all markers tested for cells isolated from all sites in Fig. 1, with 2 peaks, one being the negative control (FMO) and the other one the cells incubated with the appropriate antibody. In Supplementary Table 1, data results show the percentages of positive cells with their respective standard deviation. After the flow cytometry analysis, we could observe that the cells obtained from BM expressed the least CD90 when compared with synovial and adipose tissue cells (Supplementary Fig. 3). Among all the tissues, synovial fluid cells had a slightly higher expression of CD11α/CD18, CD45, CD79α and MHC II, but the overall positivity was low to negative nonetheless. Most of the adipose tissue cells showed the highest expression of CD105 when compared with BM and synovial fluid cells (Supplementary Fig. 3). Moreover, CD90 was also highly expressed in all three adipose tissue cells (Supplementary Fig. 3). In contrast, CD45 and CD11α/CD18 had a lower expression in all of the adipose tissue cells when compared with BM and synovial fluid cells. Neck fat and tail fat cells had a higher CD79α, CD90, CD105 and MHC II expression compared with mesenteric fat. In the case of tail fat cells, they showed the highest CD90 expression, while neck fat presented the highest expression of CD105 of all the three adipose tissue cells (Supplementary Fig. 3). Mesenteric fat tissue cells expressed the least CD11α/CD18, CD45, CD79α and MHC II compared with BM, synovial fluid, neck fat and tail fat cells. Generally, synovial fluid cells showed expression of all cell surface markers tested and was the population with the highest frequency of CD11α/CD18^{+}, CD45^{+}, CD79α^{+} and MHCII^{+} cells. Only in synovial fluid cells CD45 and MHCII positive cells had a frequency above 2% (2.033 and 3.164 respectively). CD90 and CD105 expression showed higher variability as detailed in Supplementary Table 1, ranging from 9.132% to 97.043% and from 13.22% to 84.285% respectively.

3.2. Cell differentiation

Cells were cultured with differentiation media with the objective to evaluate their capacity to go through tri-lineage (adipogenic, chondrogenic and osteogenic) differentiation. This is a characteristic

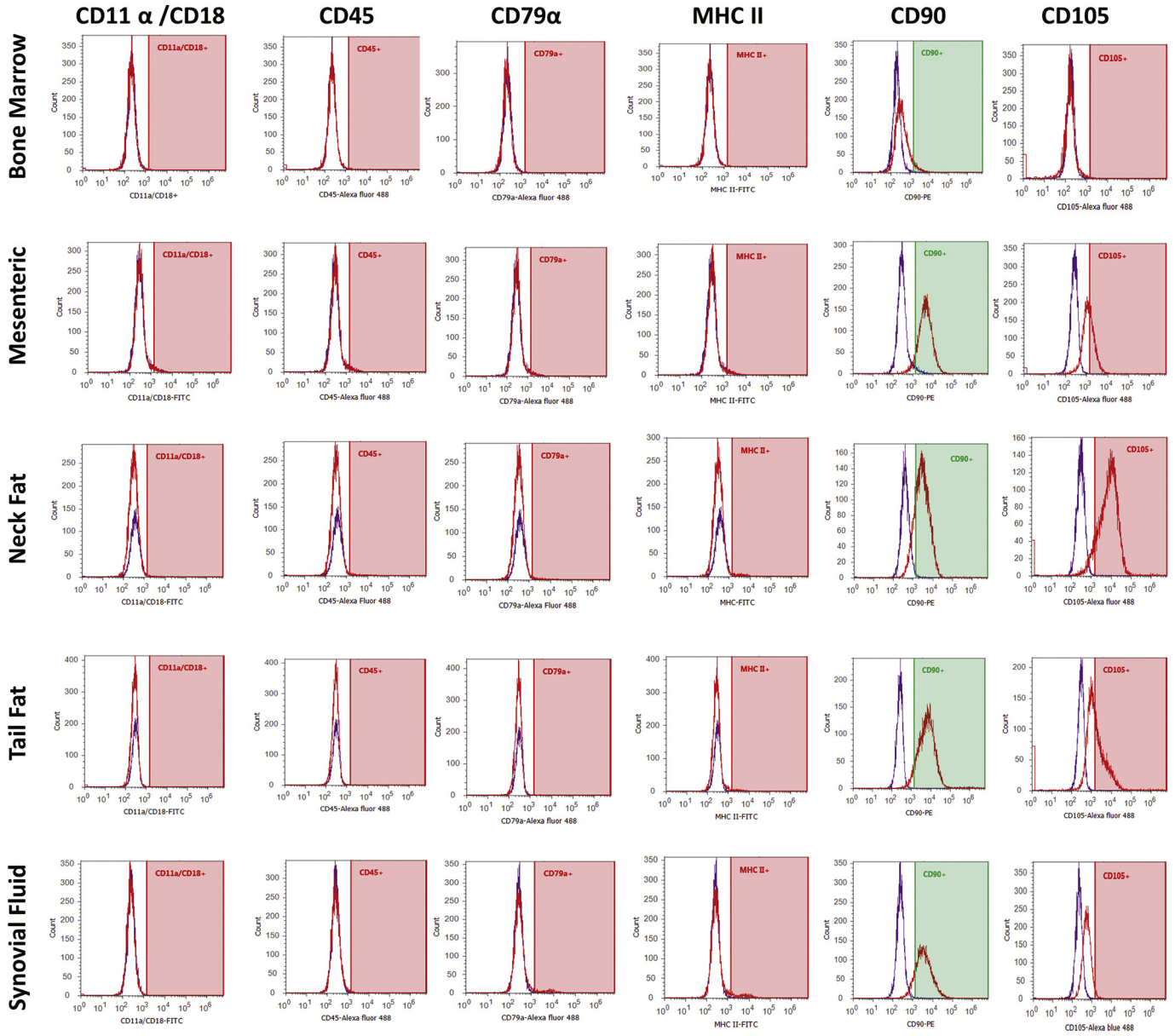


Fig. 1. Flow cytometry analysis of the different types of cells obtained from BM, synovial and adipose tissues. Each histogram displays a blue curve that corresponds to the FMO control and a red curve that shows the cells stained with the indicated antibody. Gates were drawn according to the FMO controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

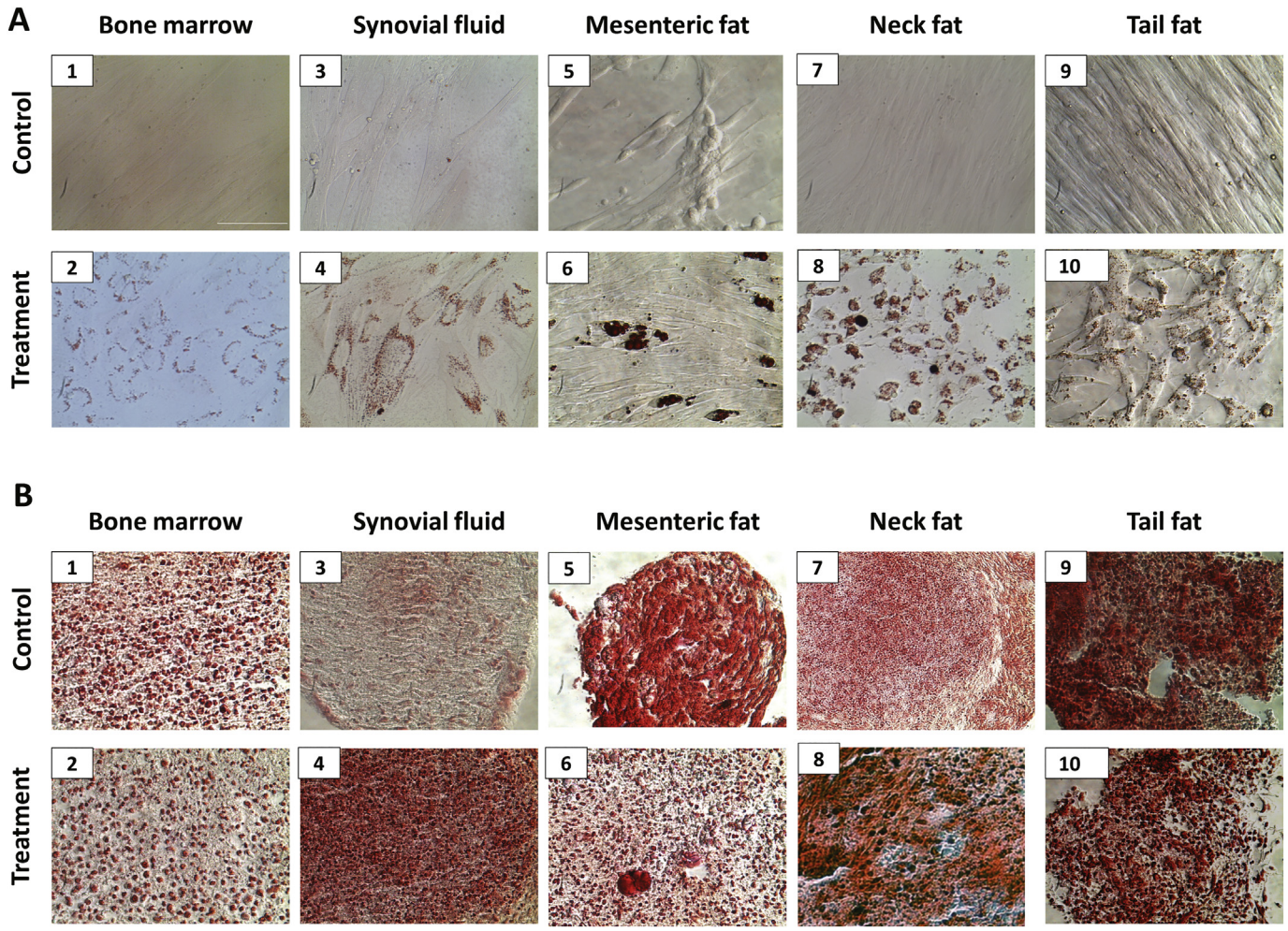


Fig. 2. Adipogenic and Chondrogenic differentiation on MSCs. A: Adipogenic differentiation after 14 days on monolayer culture with proliferation medium (Control) and differentiation medium (DM). A: Adipogenic cells were stained with Oil Red O. Panels 1-3-5-7-9 are representative pictures of cells cultured in growth medium and stained with Oil Red O. Panels 2-4-6-8-10 are the corresponding cells grown in differentiation medium and stained Oil Red O. Shape morphology differs in DM treated cells (flattened and round) *vs* controls (large cells). B: Chondrogenic differentiation was evaluated after 14 days on round bottom tubes. The cell aggregation formed a pellet that was stained with Safranin O after inclusion in paraffin and sectioning. Panels 1-3-5-7-9 are representative pictures of cells cultured in growth medium, while panels 2-4-6-8-10 are grown in differentiation medium. Treated cells with chondrogenic media show a more defined cell nuclei than control cells. Images were obtained with a 40x objective (scale bar is 50 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expected when obtaining MSCs. Adipogenic differentiation was validated by Oil Red O staining after 14 days in culture. Fig. 2A shows on the upper side the controls (with proliferation medium), whereas on the bottom a representative image of the cells in differentiation conditions has been reported. All the cells treated with differentiation medium were positively stained with Oil Red O. Control cells shape was large whereas in treated cells it was round and flattened. The Oil Red O staining allowed to observe the presence of lipid droplets within the cells that underwent adipogenic differentiation in treated cells. Overall, adipose cells showed a more intense staining when compared to BM or synovial fluid cells. Mesenteric cells (Fig. 2A6) differentiation is more evident and showed the presence of typical lipid droplets within adipose cells. For the chondrogenic differentiation, the micromass or pellet was formed. The pellet size of the chondrogenic treated cells was larger (images not show) in comparison with the controls. The control pellet aggregates did not show a defined structure and a basal Safranin O staining (Fig. 2B) was observed in the synovial and mesenteric controls (Fig. 2B3 and B5). Pellets formed by cells undergoing chondrogenesis that were stained with Safranin O (bottom images on Fig. 2B) showed a red and more defined nuclei structures when compared to controls. Osteogenic pre-differentiation was evaluated by alkaline phosphatase staining. All the controls in proliferation medium were not stained whereas on the bottom side, all the treated cells showed the positive staining for alkaline phosphatase pre-osteogenic differentiation in all the different cell types (Fig. 3A). Final osteogenic differentiation was detected by Alizarin red staining and treated cells showed irregular shape. As shown in Fig. 3B, in all the osteogenesis induced cell types positive red stained areas were detected, confirming final osteogenic differentiation by the presence of calcium deposits.

3.3. Cell migration

Migration cell capacity and motility was performed on all cells by the use of porous membrane inserts (transwells). This migration assay might give us information on the ability of cells to move through a porous membrane which could be related to their capacity to migrate *in vivo*. At 24 h, we could observe that there were significant ($p < .05$) differences in the migration capacity of BM against mesenteric and neck fat cells (Fig. 4). Within the adipose tissue cells, tail fat showed a higher tendency to migrate when compared with mesenteric and neck fat; however migration was not statistically different ($p > .05$) among cells recovered from the different adipose tissues (Fig. 4). Tail fat, BM and

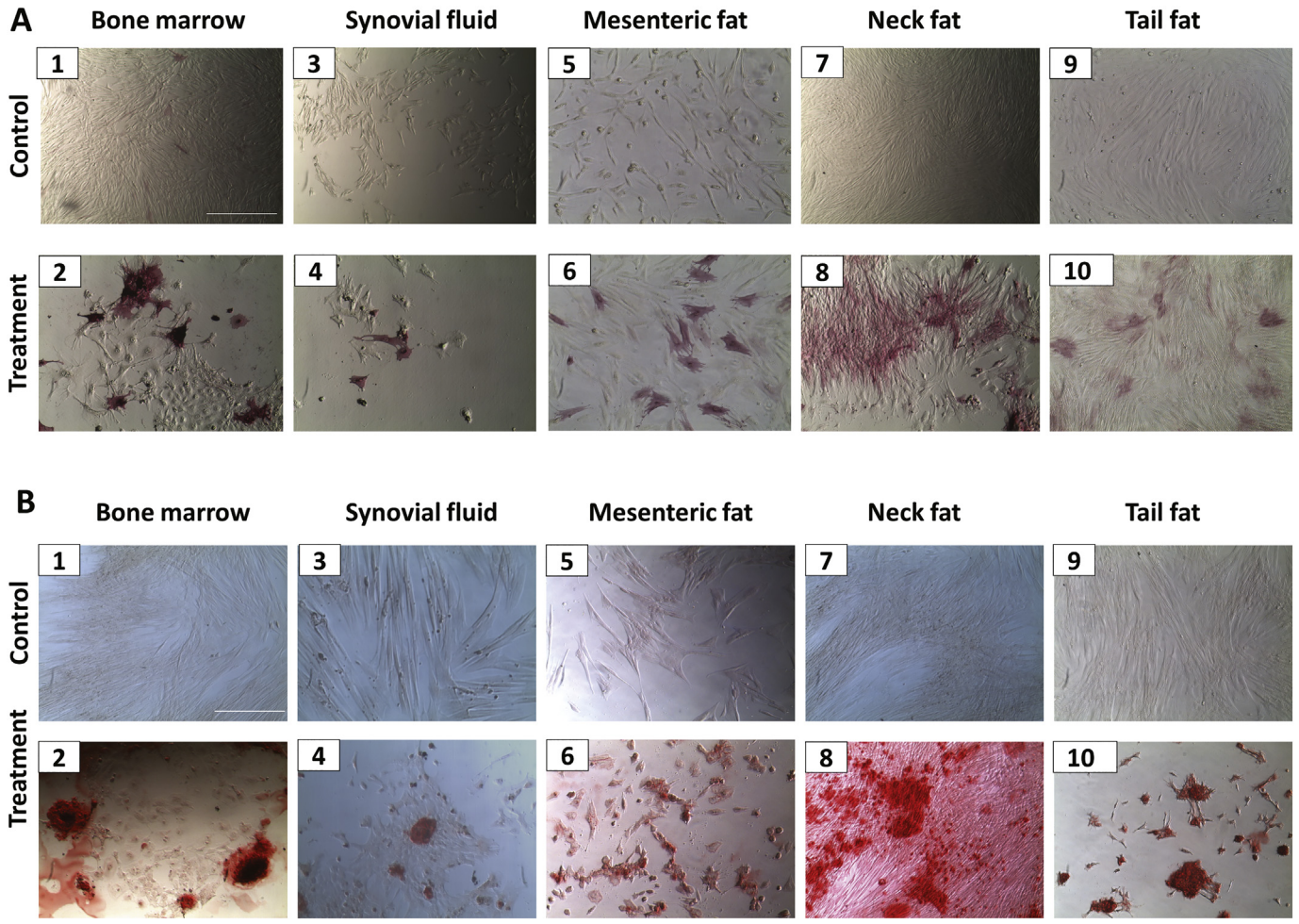
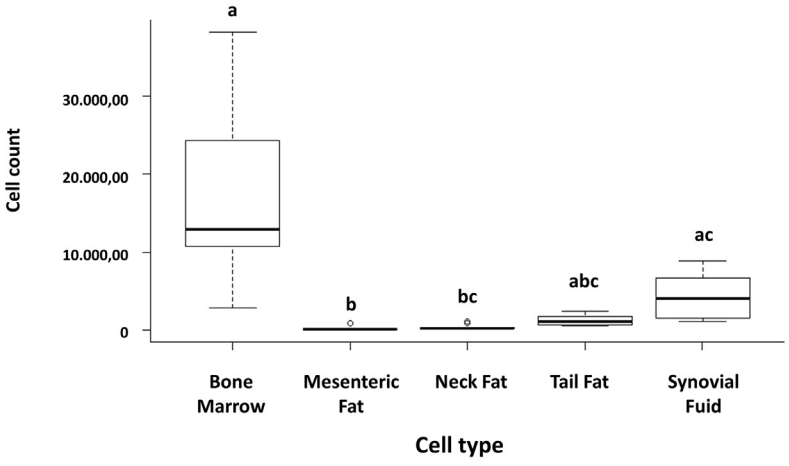


Fig. 3. Osteogenic differentiation on MSCs. A: Osteogenic differentiation was evaluated after 7 days in differentiation medium and growth medium on monolayer cultures. Alkaline phosphatase analysis was performed. Panels 1-3-5-7-9 show control cells, while panels 2-46-8-10 show cells grown in differentiation medium. Control cells looks large without specific staining whereas treated cells showed a different morphology (bigger and irregular shape) and intense red/purple staining. B: Final osteogenic differentiation was performed after around 21 days on monolayer culture. Cells were stained with alizarin red, panels 1-3-5-7-9 are representative images of controls in growth medium, while panels 2-46-8-10 are images of cells grown in differentiation medium. Osteogenic positive cells showed a red staining and a different morphological shape. Images were obtained with a 10x objective (scale bar is 250 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Letters indicate the results of post-hoc pairwise comparison. Groups identified with different letters are statistically different (p<0.05).

Fig. 4. Cell migration capacity of BM, synovial and adipose cells. Columns represent quantification of cell migration capacity as nuclei counts of the cells that were attached on the lower side of the porous membrane. Results are representative of three animal replicates, each one done in triplicate. Differences on letters above the columns indicate statistically significant differences ($p < .05$).

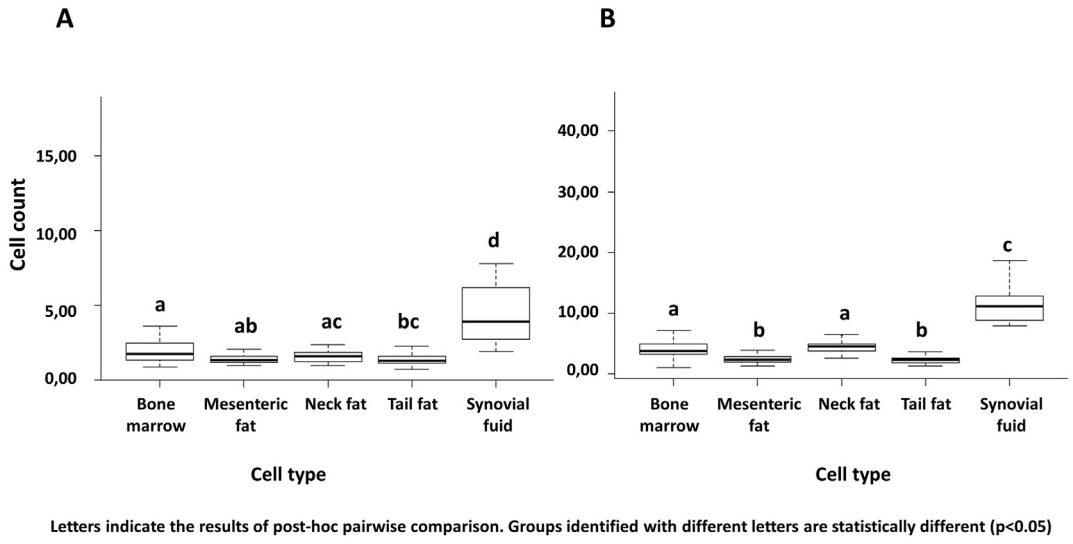


Fig. 5. Cell growth of BM, synovial and adipose cells. Columns represent quantification of cell growth as ratio of nuclei counted at a specified time point (24 h or 48 h) and nuclei counted at time 0. A: Cell growth at 24 h B: Cell growth at 48 h. Results are representative of three animal replicates. Differences on letters above the columns indicate statistically significant differences ($p < .05$).

synovial fluid were not significantly different and mesenteric and neck fat cells seem to be likely similar on their migration capacity. Synovial fluid cells showed a statistically significant higher migration capacity ($p < .05$) than mesenteric and neck fat cells.

3.4. Cell growth

To study an additional characteristic of these cells, we decided to analyze *in vitro* cell growth. Cells were plated in 6-well dishes and were evaluated at two different times (24 and 48 h). Within the 24 h in culture, BM, mesenteric and neck fat did not show significant differences ($p > .05$) (Fig. 5) in cell growth. For the cells from mesenteric and tail fat, cell growth was not significantly different ($p > .05$), this was also the case between mesenteric and neck fat ($p > .05$) (Fig. 5). Synovial fluid cells were significantly different ($p < .05$) when compared with BM and all the adipose cells, showing a higher amount of cells after 24 h in culture (Fig. 5).

When the analysis was repeated at 48 h, we could observe that BM and neck fat cells were significantly different ($p < .05$) from mesenteric, tail fat and synovial fluid cells (Fig. 5). Adipose (mesenteric and tail fat) cells were similar between them and significantly different ($p < .05$) from neck fat cells (Fig. 5). Finally, synovial fluid cells seems to grow significantly more ($p < .05$) (Fig. 5). Cell culture length seems to modify cell growth in all samples. Adipose cells growth patterns from mesenteric cells become significantly different ($p < .05$) from BM cells after 48 h in culture (Fig. 5). In both cases, synovial fluid cells remain significantly different within both 48 and 24 h in culture, presenting the highest cell growth capacity in *in vitro* cultures.

3.5. Wound healing assay

MSCs derived from BM, synovial fluid and adipose tissues were seeded in a co-culture with endothelial cells to analyze their ability to improve the wound healing capacity of a target cell population by replenishing a scratch area. After 16 h of being in culture, differences on the amount of cells that were able to migrate to the wound healing area was analyzed. We could observe that among the different type of MSCs there were not statistically significant differences ($p < .05$) with the controls (endothelial cells) when they were seeded in a co-culture system (Fig. 6). However, when conditioned medium from MSCs was used a statistically significant difference ($p < .05$) was observed as the BM cells were the only ones to improve scratch filling (Fig. 7). BM cells

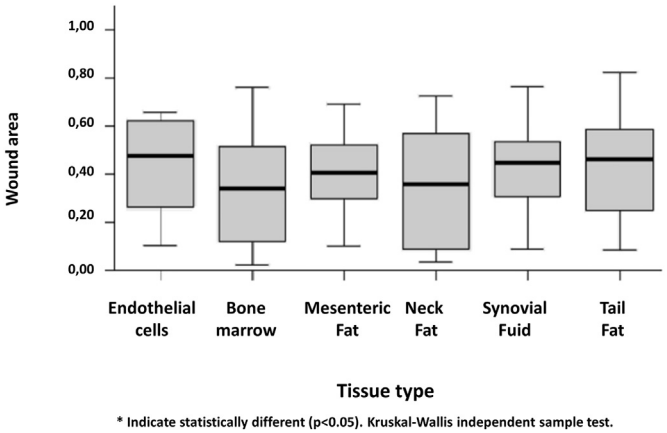


Fig. 6. Wound healing *in vitro* assay of endothelial cells in a co-culture with MSCs. Columns show the ratio between the wound area measured at 16 h and at time 0 when co-culture was performed. Each different type of mesenchymal cells was performed in replicates from each animal ($n = 3$). Asterisk (*) indicates statistically different ($p < .05$). Scale bars represent S.E.M.

were the cells that were able to largely recover wound healing areas when compared with other type of MSCs. Representative images at time 0 and 16 h are shown (Supplementary Fig. 2).

4. Discussion

We described here different MSCs obtained from BM, synovial and adipose tissues from adult equines. We were able to confirm their MSCs identity and to evaluate *in vitro* performances and capabilities of MSCs isolated from different sources. We propose here that different sources might impact MSCs properties from adult equines for putative use in cell therapy. Comparison of different MSCs sources could also prove valuable for veterinarian clinicians in case the equine's capacity as a donor might not be adequate due to age, or inadequate health condition (Bamford et al., 2014; Carter-Arnold et al., 2014; Schlueter and Orth, 2004). Such information would allow clinicians to select for an alternative source. When we obtained these cells after several days of *in vitro* culture, we were able to observe the typical MSCs fibroblast-like spindle morphology in our cells (De Schauwer et al., 2011). However, in BM cells we observed different cellular morphology which reflect their

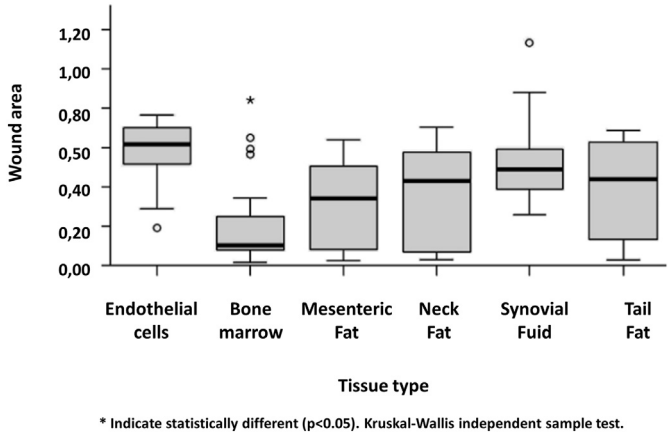


Fig. 7. Wound healing *in vitro* assay of endothelial cells with conditioned medium from MSCs Columns show the ratio between the wound area measured at 16 h and at time 0 when conditioned medium derived from MSCs was used. Each conditioned medium was derived from each type of MSCs cell and replicates were made from each animal (n = 3). Asterisk (*) indicates a statistically significant difference ($p < .05$). Scale bars represent S.E.M.

heterogeneous population (Elsafadi et al., 2016). Other studies have evaluated cells obtained from different sources *i.e.* BM and adipose tissue (Ranera et al., 2011) however, tissue collection areas were different in our study. For instance, adipose tissue is mostly obtained from the tail base region (Ranera et al., 2011) but adipose cells (from the tail base) obtained from equines with metabolic syndrome decrease in their potency, proliferation and differentiation capacity when compared with healthy adipose cells (Marycz et al., 2016b). We therefore further compared cells derived from other adipose tissues. We selected mesenteric fat because of its ease of processing and its reported high cell viability (Metcalf et al., 2016). We also decided to obtain synovial fluid cells from slaughtered animals to evaluate their mesenchymal nature and culture properties since there are reports of such cells isolated from synovial fluid of live equines (Murata et al., 2014). Since MSCs have been studied for transplantation purposes (Ortved and Nixon, 2016; Schlueter and Orth, 2004), our results show that BM followed by synovial fluid cells possess a higher migration capacity than other sources. Moreover, cells obtained from synovial fluid have a higher cell growth *in vitro*.

When performing the phenotype analysis on equine MSCs, we decided to be consistent with the minimal criteria that have been defined by Dominici et al. (Dominici et al., 2006), so we analyzed the expression of specific markers associated both with an MSCs phenotype and a non-MSCs phenotype. Such criteria would demand a frequency lower than 2% for $CD45^+$, $CD11\alpha/18^+$, $CD79\alpha^+$, $MHCII^+$ cells and higher than 95% for $CD90^+$ and $CD105^+$ cells. However, such frequencies are defined for human MSCs and in other species variations can be expected as different frequencies have been reported for the equine species (Paebst et al., 2014). We could find a low percentage of $CD45^+$ cells in synovial fluid, just above the threshold. We hypothesize that this expression could possibly relate to the sampling site and procedure. A low percentage of synovial fluid cells expressed MHC-II. The low frequency of MHC-II^+ cells in our study is in concordance with other studies (Murata et al., 2014), and MHC-II expression might be limited to early passages and decreasing in later passages (Radcliffe et al., 2010). However, MSCs heterogeneity and MHC-II expression differ in the equine species according to age as reported (Schnabel et al., 2014). Moreover, a very low frequency of MHC-II^+ cells is important for the MSCs value in cell therapy (Schnabel et al., 2014) since MHC-II^+ cells could trigger an immune response.

Thus, the low, albeit above threshold, frequency of MHC-II^+ cells present in synovial fluid in our study suggest that MSCs obtained from this tissue should be carefully considered for allogeneic transplantation.

According to defined criteria, MSCs should express CD90 and CD105 (De Schauwer et al., 2011; Dominici et al., 2006). The $CD90^+$ cells in mesenteric, neck and tail fat cells are well documented in equine MSCs and serve as "identifiers" of these type of cells (Ranera et al., 2011). Consistent with other studies (Metcalf et al., 2016), our data show that in tail and neck fat, $CD90^+$ cells were detected. However, our data show a lower CD90 expression in mesenteric cells, compared to that of tail and neck fat cells. This could be explained by our methodological analysis, since we analyzed CD90 by flow cytometry while Metcalf et al. (Metcalf et al., 2016) measured gene expression. Another possible explanation of this is that the sampling collection site might influence mesenteric cell type CD90 expression, since mesentery is a large tissue. $CD90^+$ cell frequency in synovial fluid and BM cells in our study were consistent with other experiments (Paebst et al., 2014; Prado et al., 2015). In our study, CD105 expression was found in all types of MSCs including adipose-derived cells even though at frequency lower than 95%. Even though a high frequency of expression is required, our data are consistent with similar CD105 expression in equine adipose (Paebst et al., 2014) and synovial fluid cells (Prado et al., 2015). CD105 has been used to characterize MSCs with a possible chondrogenic potential due to its relationship with the transforming growth factor beta (TGF-β) complex which stimulates chondrogenic differentiation (Cleary et al., 2016). However, CD105 expression by itself should not be directly associated with chondrogenic potential in cells (Cleary et al., 2016). We not only found the surface marker expression to be consistent with previous reports, but we also confirmed the MSCs identity through tri-lineage differentiation. Mesenteric fat cells showed a more evident lipid droplet content with Oil Red O staining, which might be related to visceral tissues having a more lipolitical activity when compared to other adipose tissues, like subcutaneous fat (Bjorndal et al., 2011). Among all of the tissues, synovial fluid cells showed the highest Safranin O staining and a more defined cell morphology. This might depend on their origin being closer to a chondrogenic differentiation fate, as it has been prevously described by Zayed et al. (Zayed et al., 2017). It has been observed that synovial fluid cells improve regeneration by hyaline cartilage production and reconstruct the defected area when implanted *in vivo* in murine articulations (Zayed et al., 2018b). BM, synovial and adipose cells were able to differentiate into osteocytes. The higher or lower differentiation potential in cells might vary among donors (Zayed et al., 2017). Moreover, in the case of equine synovial fluid cells, even if they might represent a less invasive MSCs harvesting site in equines, there is still the challenge of further evaluating their use in intra-articular injections *i.e.* osteoarthritis to support their clinical application (Zayed et al., 2018a).

The highest BM cell migration reported herein could relate to a higher motility and infiltration capacity (Smith et al., 2012). In our study, cell migration was evaluated at the first passage. BM cells results, followed by synovial fluid cells, in the migration assay might depend on their efficiency to move through sites of injury and thus influence their progenitor/stemness and environment interaction (Augello et al., 2010). Their migration ability might also depend to a varying extent to the extracellular matrix they were isolated from. As for adipose-derived cells, their cell migration capacity is not extensively developed (Cabezas et al., 2018).

Cell growth is a meaningful characteristic to consider when using MSCs for cell therapy, because cell expansion is needed in order to obtain a significant amount of cells for transplantation. After evaluating *in vitro* cell growth we might suggest that synovial fluid cells possess an adequate cell expansion in culture. MSCs isolated from equines suffering joint diseases have a higher proliferation rate when compared with synovial fluid cells from healthy equines (Murata et al., 2014). However, if the implantation of MSCs from healthy horses resulted in an increased inflammatory response (Ardanaz et al., 2016), such an effect might even be higher when using MSCs from diseased joints and therefore more studies should focus on this issue. In our study, the

higher growth rate of synovial fluid cells might be related to the type of stem/progenitor cells contained within the tissue niche (Prado et al., 2015). When comparing BM against adipose-derived cells in our study, we could observe that neck fat cells had an almost similar cell growth at 24 and 48 h.

In vitro wound healing assay in a co-culture with endothelial and MSCs did not show significant differences. Neck fat MSCs efficiency to refill scratch area has been reported on adipose equine cells (Cabezas et al., 2018) obtained from the rump region (tail fat). However, in our study we could observed that neck fat cells were more effective in wound healing repair than tail fat cells. Only when adding conditioned media from BM derived MSCs to the *in vitro* cultures, we detected a statistically significant improvement in wound repair. Our results suggest that BM cells might release some paracrine factors (*i.e.* chemokines) that enhance scratch repair (Smith et al., 2012) and that this secretion occurs at low rate, so that an extended time period is necessary to have the necessary accumulation that results in a biological effect, hence the enhancement of wound healing repair when conditioned medium was used *vs* co-cultures.

The tissue cell sources in our study provide a more extended overview of MSCs compared characteristics different from the typically BM and adipose tail fat cells largely described in equines literature.

5. Conclusion

We demonstrate that MSCs from BM, synovial fluid and adipose (mesenteric, neck and tail fat) tissue from adult slaughtered equines could be valuable candidates as an allogeneic source for cell therapy, although they show different phenotypes and properties. All cell types were able to grow on monolayer cultures, to differentiate into the trilineage adipogenesis, chondrogenesis and osteogenesis, confirmed by all their MSCs characteristics. In the case of BM cells, we could observe that they possess an important migration capacity and might be useful during implantation. Media obtained from BM cell cultures might contain molecules that enhance wound healing repair more effectively than the media from other MSCs. In contrast, synovial fluid cells are a good source for obtaining MSCs in a shorter time due to their cell growth and they might be suggested as a source of rapidly expanding MSCs. Phenotype and performances of each different MSCs source should then be considered when making a choice in selecting MSCs. In some case, higher proliferation rates might be more desirable to reduce the time between sampling and transplantation, while in others the more important property might be a higher paracrine effect. Our data might help veterinarian clinicians decide which source is more convenient and appropriate for the condition of each individual patient.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101442.

Author disclosure statement

No competing financial interests exist.

CRediT authorship contribution statement

Magdalena Arévalo-Turrubiarte: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Chiara Olmeo:** Investigation. **Paolo Accornero:** Methodology, Formal analysis. **Mario Baratta:** Formal analysis, Supervision, Writing - original draft. **Eugenio Martignani:** Conceptualization, Formal analysis, Supervision, Writing - original draft, Writing - review & editing.

Acknowledgements

We thank Dr. Walid Azab from the Free University of Berlin, Institut für Virologie Robert von Ostertag-Haus-Zentrum für Infektionsmedizin, Germany for the equine endothelial (carotid artery) cells. We also thank Cristina Cecere for her technical assistance in the laboratory.

References

Ardanaz, N., Vázquez, F.J., Romero, A., Remacha, A.R., Barrachina, L., Sanz, A., Ranera, B., Vitoria, A., Albareda, J., Prades, M., Zaragoza, P., Martín-Burriel, I., Rodellar, C., 2016. Inflammatory Response to the Administration of Mesenchymal Stem Cells in an Equine Experimental Model: Effect of Autologous, and Single and Repeat Doses of Pooled Allogeneic Cells in Healthy Joints. https://doi.org/10.1186/s12917-016-0692-x.

Augello, A., Kurth, T., De Bari, C., 2010. Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. Eur. Cells Mater. 20, 121–133. https://doi.org/10.22203/eCM.v020a11.

Bamford, N.J., Potter, S.J., Harris, P.A., Bailey, S.R., 2014. Breed differences in insulin sensitivity and insulinemic responses to oral glucose in horses and ponies of moderate body condition score. Domest. Anim. Endocrinol. 47, 101–107. https://doi.org/10.1016/J.DOMANIEND.2013.11.001.

Bjorndal, B., Burri, L., Staalesen, V., Skorve, J., Berge, R.K., 2011. Different adipose depots: their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents. J. Obes. 2011, 490650. https://doi.org/10.1155/2011/490650.

Cabezas, J., Rojas, D., Navarrete, F., Ortiz, R., Rivera, G., Saravia, F., Rodriguez-Alvarez, L., Castro, F.O., 2018. Equine mesenchymal stem cells derived from endometrial or adipose tissue share significant biological properties, but have distinctive pattern of surface markers and migration. Theriogenology 106, 93–102. https://doi.org/10.1016/J.THERIOGENOLOGY.2017.09.035.

Carter-Arnold, J.L., Neilsen, N.L., Amelse, L.L., Odoi, A., Dhar, M.S., 2014. In vitro analysis of equine, bone marrow-derived mesenchymal stem cells demonstrates differences within age- and gender-matched horses. Equine Vet. J. 46, 589–595. https://doi.org/10.1111/evj.12142.

Cleary, M.A., Narcisi, R., Focke, K., van der Linden, R., Brama, P.A.J., van Osch, G.J.V.M., 2016. Expression of CD105 on expanded mesenchymal stem cells does not predict their chondrogenic potential. Osteoarthr. Cartil. 24, 868–872. https://doi.org/10.1016/j.joca.2015.11.018.

Colleoni, S., Bottani, E., Tessaro, I., Mari, G., Merlo, B., Romagnoli, N., Spadari, A., Galli, C., Lazzari, G., 2009. Isolation, growth and differentiation of equine mesenchymal stem cells: effect of donor, source, amount of tissue and supplementation with basic fibroblast growth factor. Vet. Res. Commun. 33, 811–821. https://doi.org/10.1007/s11259-009-9229-0.

De Schauwer, C., Meyer, E., Van de Walle, G.R., Van Soom, A., 2011. Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. Theriogenology 75, 1431–1443. https://doi.org/10.1016/j.theriogenology.2010.11.008.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., Horwitz, E., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315–317. https://doi.org/10.1080/14653240600855905.

Elsafadi, M., Manikandan, M., Atteya, M., Hashmi, J.A., Iqbal, Z., Aldahmash, A., Alfayez, M., Kassem, M., Mahmood, A., 2016. Characterization of cellular and molecular heterogeneity of bone marrow stromal cells. Stem Cells Int. 2016https://doi.org/10.1155/2016/9378081. 9378081.

Friedenstein, A.J., Piatetzky-Shapiro, I., Petrakova, K.V., 1966. Osteogenesis in transplants of bone marrow cells. Embryol. Exp. Morph. 16 581–390.

Hipp, J., Atala, A., 2008. Sources of stem cells for regenerative medicine. Stem Cell Rev. 4, 3–11. https://doi.org/10.1007/s12015-008-9010-8.

Jurgens, W.J.F.M., Oedayrajsingh-Varma, M.J., Helder, M.N., ZandiehDoulabi, B., Schouten, T.E., Kuik, D.J., Ritt, M.J.P.F., van Milligen, F.J., 2008. Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies. Cell Tissue Res. 332, 415. https://doi.org/10.1007/s00441-007-0555-7.

Kornicka, K., Marycz, K., Maredziak, M., Tomaszewski, K.A., Nicpon, J., 2017. The effects of the DNA methyltranfserases inhibitor 5-Azacitidine on ageing, oxidative stress and DNA methylation of adipose derived stem cells. J. Cell. Mol. Med. 21, 387–401. https://doi.org/10.1111/jcmm.12972.

Lombana, K.G., Goodrich, L.R., Phillips, J.N., Kisiday, J.D., Ruple-Czerniak, A., McIlwraith, C.W., 2015. An investigation of equine mesenchymal stem cell characteristics from different harvest sites: more similar than not. Front. Vet. Sci. 2, 67. https://doi.org/10.3389/fvets.2015.00067.

Marycz, K., Kornicka, K., Grzesiak, J., Smieszek, A., Szlapka, J., 2016a. Macroautophagy and selective mitophagy ameliorate chondrogenic differentiation potential in adipose stem cells of equine metabolic syndrome: new findings in the field of progenitor cells differentiation. Oxidative Med. Cell. Longev. 2016, 3718468. https://doi.org/10.1155/2016/3718468.

Marycz, K., Kornicka, K., Maredziak, M., Golonka, P., Nicpon, J., 2016b. Equine metabolic syndrome impairs adipose stem cells osteogenic differentiation by predominance of autophagy over selective mitophagy. J. Cell. Mol. Med. 20, 2384–2404. https://doi.org/10.1111/jcmm.12932.

Metcalf, G.L., McClure, S.R., Hostetter, J.M., Martinez, R.F., Wang, C., 2016. Evaluation of adipose-derived stromal vascular fraction from the lateral tailhead, inguinal region, and mesentery of horses. Can. J. Vet. Res. 80, 294–301.

Murata, D., Miyakoshi, D., Hatazoe, T., Miura, N., Tokunaga, S., Fujiki, M., Nakayama, K., Misumi, K., 2014. Multipotency of equine mesenchymal stem cells derived from synovial fluid. Vet. J. 202, 53–61. https://doi.org/10.1016/j.tvjl.2014.07.029.

Ortved, K.F., Nixon, A.J., 2016. Cell-based cartilage repair strategies in the horse. Vet. J. 208, 1–12. https://doi.org/10.1016/j.tvjl.2015.10.027.

Paebst, F., Piehler, D., Brehm, W., Heller, S., Schroeck, C., Tárnok, A., Burk, J., 2014. Comparative immunophenotyping of equine multipotent mesenchymal stromal cells: an approach toward a standardized definition. Cytom. Part A 85, 678–687. https://doi.org/10.1002/cyto.a.22491.

Prado, A.A.F., Favaron, P.O., da Silva, L.C.L.C., Baccarin, R.Y.A., Miglino, M.A., Maria, D.A., 2015. Characterization of mesenchymal stem cells derived from the equine synovial fluid and membrane. BMC Vet. Res. 11, 281. https://doi.org/10.1186/s12917-015-0531-5.

Radcliffe, C.H., Flaminio, M.J., Fortier, L.A., 2010. Temporal analysis of equine bone marrow aspirate during establishment of putative mesenchymal progenitor cell populations. Stem Cells Dev. 19, 269–282. https://doi.org/10.1089/scd.2009.0091.

Ranera, B., Lyahyai, J., Romero, A., Vázquez, F.J., Remacha, A.R., Bernal, M.L., Zaragoza, P., Rodellar, C., Martín-Burriel, I., 2011. Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue. Vet. Immunol. Immunopathol. 144, 147–154. https://doi.org/10.1016/j.vetimm.2011.06.033.

Requicha, J.F., Viegas, C.A., Albuquerque, C.M., Azevedo, J.M., Reis, R.L., Gomes, M.E., 2012. Effect of anatomical origin and cell passage number on the stemness and osteogenic differentiation potential of canine adipose-derived stem cells. Stem Cell Rev. 8, 1211–1222. https://doi.org/10.1007/s12015-012-9397-0.

Schlueter, A.E., Orth, M.W., 2004. Equine osteoarthritis: a brief review of the disease and its causes. Equine Comp. Exerc. Physiol. 1, 221–231. https://doi.org/10.1079/ECP200428.

Schnabel, L.V., Pezzanite, L.M., Antczak, D.F., Felippe, M.J., Fortier, L.A., 2014. Equine bone marrow-derived mesenchymal stromal cells are heterogeneous in MHC class II expression and capable of inciting an immune response in vitro. Stem Cell Res. Ther. 5, 13. https://doi.org/10.1186/scrt402.

Smith, H., Whittall, C., Weksler, B., Middleton, J., 2012. Chemokines stimulate bidirectional migration of human mesenchymal stem cells across bone marrow endothelial cells. Stem Cells Dev. 21, 476–486. https://doi.org/10.1089/scd.2011.0025.

Woeller, C.F., O'Loughlin, C.W., Pollock, S.J., Thatcher, T.H., Feldon, S.E., Phipps, R.P., 2015. Thy1 (CD90) controls adipogenesis by regulating activity of the Src family kinase. Fyn. FASEB J. 29, 920–931. https://doi.org/10.1096/fj.14-257121.

Zayed, M., Caniglia, C., Misk, N., Dhar, M.S., 2017. Donor-matched comparison of chondrogenic potential of equine bone marrow- and synovial fluid-derived mesenchymal stem cells: implications for cartilage tissue regeneration. Front. Vet. Sci. 3, 121. https://doi.org/10.3389/fvets.2016.00121.

Zayed, M., Adair, S., Ursini, T., Schumacher, J., Misk, N., Dhar, M., 2018a. Concepts and challenges in the use of mesenchymal stem cells as a treatment for cartilage damage in the horse. Res. Vet. Sci. 118, 317–323. https://doi.org/10.1016/j.rvsc.2018.03.011.

Zayed, M., Newby, S., Misk, N., Donnell, R., Dhar, M., 2018b. Xenogenic implantation of equine synovial fluid-derived mesenchymal stem cells leads to articular cartilage regeneration. Stem Cells Int. 2018, 1–9. https://doi.org/10.1155/2018/1073705.

Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P., Hedrick, M.H., 2002. Human adipose tissue is a source of multipotent stem cells. Mol. Biol. Cell 13, 4279–4295. https://doi.org/10.1091/mbc.E02-02-0105.