doi: 10.4149/gpb_2021026

Impact of mesenchymal stem cells derived conditioned media on neural progenitor cells

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Abstract. Neurodegenerative diseases are common problem for companion animals. Due to the limited ability of injured axons to regenerate, innovative therapies combined with rehabilitation have been applied and evaluated. Among them, stem cells and their conditioned media implantation, which can ameliorate damaged tissue has been suggested as a promising treatment strategy. The main goal of our study was to characterize mesenchymal stem cells (MSC) derived from canine adipose tissue (AT-MSC) and umbilical cord (UC-MSC) and analyse effect of their conditioned media (CM) on neurite outgrowth of neural progenitor cells isolated from the brain cortex of neonatal rats. MSC from both sources showed high osteogenic and chondrogenic potential and expression of CD90 and CD29. Furthermore, both UC-MSCCM and AT-MSCCM stimulated neurite growth. Interestingly, this effect was more pronounced with UC-MSCCM when compared to AT-MSCCM *in vitro*, which may be related to the different content of neurotrophic factors included in the CM.

Key words: Neural progenitor cells — Mesenchymal stem cells — Umbilical cord — Adipose tissue neurite growth

Introduction

Neural progenitor cells (NPCs) are multipotent cells that are able to generate four major cell types of the central nevous system (CNS): astrocytes, oligodendrocytes, microglia and neurons. NPCs are characterized by multilineage potency and self-renewal capacity during embryonic development and they can be found also in neurogenic regions of the adult CNS (Altman and Das 1965). In addition, the adult spinal cord has been shown to contain NPCs in the white matter parenchyma (Horner et al. 2000; Yamamoto et al. 2001) and in the region around the central canal (Kulbatski and Tator 2009), either in the ependyma (Meletis et al. 2008) or subependymally (Martens et al. 2002).

Mesenchymal stem cells (MSC) are a subset of stromal cells that maintain the same fibroblast-like morphology and specific cluster of differentiation (CD) marker expression; however they also have the potential for self-renewal and ability to differentiate into adipocytes, chondrocytes, and osteoblasts *in vitro* (Horwitz et al. 2005). Cultured cells can be classified as MSC if they show: (1) adherence to plastic under culture conditions, (2) expression of CD105, CD73, and CD90, (3) lack of expression of CD45, CD34, CD14/

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CD11b, CD79/CD19, and HLA-DR surface markers, and (4) multilineage potential (Dominici et al. 2006). Compelling evidence exists that non-haematopoietic stem cells, including MSC, exert a substantial beneficial and therapeutic effect after transplantation in experimental CNS disease models through the secretion of immune-modulatory or neurotrophic paracrine factors (Hofer and Tuan 2016).

The stem cell-derived secreted factors including cytokines, chemokines and growth factors have gained increasing attention in recent years because of their multiple implications for the repair, restoration or regeneration of injured tissues (Kang et al. 2014). The secreted factors can be found in the medium in which the stem cells are cultured; referred as conditioned medium (CM).

The use of CM has several advantages compared to the use of stem cells, since CM can be manufactured, freeze-dried, packaged, and transported more easily. Moreover, as it is devoid of cells there is no need to match the donor and the recipient to avoid rejection (Pawitan 2014). CM released by cells contain a range of membrane-enclosed extracellular vesicles (EV). Among them, exosomes and microvesicles carry proteins, signaling lipids, and nucleic acids (mRNA, miRNA) from donor cells to recipient cells, and thus have been proposed to serve as intercellular mediators of communication (Deng et al. 2018). Especially exosomes, membrane-enclosed nanovesicles of 30 to 150 nm that shuttle active cargoes between different cells have received a lot of attention. Exosomes, contrary to microvesicles, are formed within multivesicular bodies (MVB) at the endolysosomal pathway and are secreted upon fusion of MVB with the plasma membrane (Kalani et al. 2014).

The aim of our study was to isolate and characterize MSC derived from umbilical cord (UC-MSC) and adipose tissue (AT-MSC), from a morphological point of view, multilineage potential and expression of CD markers (CD29, CD90, CD45) according to the criteria of International Society for Stem Cells Research (ISSCR) for MSC. Subsequently, we prepared conditioned medium from both populations of MSC and compared their impact on neurite outgrowth of NPCs.

Material and Methods

Animals

The study was performed with approval and in accordance to the guidelines of the Institutional Animal Care and Use Committee of the Slovak Academy of Sciences and with the European Communities Council Directive (2010/63/EU) regarding the use of animals in research, Slovak Law for Animal Protection No. 377/2012 and 436/2012 and protocol approval Ro-4081/17-221.

Subcutaneous fat was obtained under local anesthesia (Lidocain 2% a.u.v, Biopharm, Czech Republic) from dorsal scapular region of thoroughbred dogs (n = 3); German Shepherd (3 years old, 36 kg), Royal Poodle (3 years old, 29 kg) and Slovak Čuvač (4 years old, 45 kg).

Umbilical cord was obtained *via* caesarean section (CS) under general anesthesia (Sevohale 100% v/v, Chanele Pharmaceuticals Manufacturing, Ireland), (n = *2*) from newborns of German Rotweiler (weight of a newborn was approximately 455 g, number of puppies following CS was 2) and German Doberman (weight of a newborn was approximately 370 g, n = *6*).

All these procedures were practised after clinical examination and obtaining the informed consent from their owners.

Isolation, cultivation of MSC and preparation of conditioned media

AT-MSC, UC-MSC isolation procedure

The adipose tissue (5–7 g) and umbilical cord (4–6 g), (n = 3) were washed extensively with phosphate buffer saline (PBS) with 2% antibiotic-antimycotic solution containing penicillin, streptomycin, and amphotericin B (Biowest, USA). Mechanically dissected tissue was then enzymatically dissociated with 0.05% Collagenase type I (Gibco, USA) under gentle agitation for 30–45 min at 37°C. After incubation, digested tissue was filtered (suspension was passed through a 100-μm cell strainer) to remove the rest of tissue fragments. Obtained fractions of MSC from both sources were centrifuged at 400 × g/10 min. The pellets of stromal vascular fraction (SVF) and pellets which contained isolated MSC from umbilical cord were resuspended in alpha MEM medium (Biowest, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest, USA) and 1% antibiotics (penicillin/streptomycin, Biowest, USA), and plated on 25 cm^2 tissue culture flask. Non-adherent cells were removed after 2–3 days; culture media was changed twice a week.

Flow cytometry with canine CD markers

Canine AT-MSC and UC-MSC from passage 3 were sampled to investigate presence of the CD29 and CD90-positive and CD45-negative cells. Each suspension of cells (1×10^6/ml) was incubated with fluorochrome-conjugated monoclonal antibodies: anti-CD45/FITC, anti-CD29/R-phycoerythrin, anti-CD90/allophycocyanin, diluted in PBS (MP Biomedicals, France) for 45 min at room temperature and in the dark. After incubation, the cells were washed twice with 1 ml PBS (MP Biomedicals, France), followed by 5 min centrifugation at 250 × g. Finally, 100 μl PBS were added and cytometric analysis was performed on a BD FACSCanto™ flow cytometer (Becton Dickinson Biosciences, USA) equipped with a blue (488 nm) and a red (633 nm) laser and six fluorescence detectors. The percentage of cells expressing the individual CD characters was determined by means of dot plotting for the

respective fluorescence. The data obtained were analyzed in the BD FACS DivaTM analysis software. For flow cytometry, the following antibodies were employed according to the supplier's recommendations: phycoerythrin anti-human CD29/IgG1 (Clone: TS2/16, human, canine, Sony Biotechnology); FITC anti-dog CD45/IgG2b (Clone: YKIX716.13, BIOPORT, CZ); allophycocyanin anti-dog CD90/IgG2b (Clone: YKIX337.217, BIOPORT, CZ); and their isotype controls: FITC dog IgG (CD29) and phycoerythrin, allophycocyanin dog IgG2b (CD45, CD90) from Biolegend.

Three-lineage profile of AT-MSC and UC-MSC (osteogenic, chondrogenic and adipogenic phenotypes)

The multilineage potential of canine AT-MSC and UC-MSC (passages 3) was determined by incubation with commercial StemPro Differentiation Kits containing all the reagents required for inducing canine AT-MSC and UC-MSC into chondrogenic, osteogenic, and adipogenic lineages. Cultures were stimulated with the appropriate differentiation medium for 21 days according to the recommended differentiation protocol for each specific lineage. Afterward, the cultures were fixed with 4% formaldehyde and stained with the following reagents: adipogenic culture with Oil Red, osteogenic culture with Alizarin Red S, and chondrogenic culture with Alcian Blue (all from Sigma-Aldrich, USA).

Conditioned media preparation AT-derived and UC-derived conditioned media

AT-MSCCM and UC-MSCCM at passage 3 were cultured in Dulbecco's Modified Eagle Medium (DMEM, Biowest, USA) without FBS (Biowest, USA) and antibiotics. After 24 h incubation in a humidified atmosphere with 5% CO_2 at 37°C, collected media samples were centrifuged at 400 × *g* for 10 min to remove cell debris, and filtered through a 0.2 µm sterile syringe filter (Millipore, USA). We have used identical procedure as published recently (Humenik et al. 2019). After obtaining conditioned media from both sources, the protein concentration of the CM was quantified by Bradford protein assay, using standard Bradford reagent (Sigma), to ensure that equal concentrations (1.0 mg/ml) of CM were used. DMEM was regarded as a control (nonconditioned medium). Samples of AT-MSCCM and UC-MSCCM were collected and stored at –80°C until the time of use.

Primary culture of brain cortex NPCs

NPCs were isolated from the brain cortex of three days old neonatal Wistar rats ($n = 3$). Animals were anesthetised on ice and afterwards sacrificed by decapitation. The entire brain cortex was removed, meninges were dissected away and brain cortex tissue was minced with sterile microsurgical scissors into small pieces and mechanically dissociated. Cell suspension was centrifuged 8 min at 400 × *g*. Cells were plated in 6 well plates (3×10^5 cells *per* well) and in 24 well plates (15×10^3 cells *per* well) grown in complete proliferative culture media (CPCM) composed of DMEM and Ham's F12 (DMEM-F12, Biosera, Philipines) supplemented with 5 mg/ml streptomycin, 5 IU/ml penicillin (Biochrom, UK), B27 (10 ng/ml), N2 (10 ng/ml) (Gibco, USA), bFGF (basic fibroblast growth factor) (20 ng/ml) (Milipore, USA), EGF (epidermal growth factor) (20 ng/ml) (AppliChem, Germany), 3% FBS, at 37°C in 5% CO_2 incubator for 4 days *in vitro* (DIV4).

Immunohistochemistry characterization of NPCs culture

DIV4 cells were fixed with 4% paraformaldehyde for 15 min and incubated with Anti-neurofilament 200 antibody (NF200, rabbit polyclonal IgG, Merck, USA) and Anti-glial fibrilary acidic protein (GFAP, rabbit polyclonal; Dako, USA). Appropriate secondary antibodies FITC (green goat anti-rabbit, Molecular probes, USA) were used. The cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma, USA) to reveal nuclei. The staining was detected by fluorescent microscopy (Zeiss, Germany) and pictures were taken by microscope camera (Zeiss Axiocam ERc 5s, Zeiss, Germany).

Quantification of neurite outgrowth

After 4 days, when primary culture of NPCs revealed typical neurosphere-like structures and reached 60–70% sub-confluence, cells were treated with 0.25% trypsin, and replated in 24-well plates (15×10^3 cells *per* well) for neurite growth.

NPCs were cultivated in DMEM with 5% FBS and 1% ATB for 2 days in order to adhere to plastic. Afterwards the medium was replaced to: i) AT-MSCCM ($n = 3$), ii) UC-MSCCM ($n = 3$), iii) negative CTR (control) medium (DMEM no supplements, $n = 3$) and iv) positive CTR (DMEM supplemented with B27 (10 ng/ml), $n = 3$), N2 (10 ng/ml), recombinant human bFGF (20 ng/ml) and human EGF (20 ng/ml)) and cultured for three days. The number of processes was quantified in each condition at day 1, day 2 and day 3 of culture in positive control, negative control, AT-MSCCM and UC-MSCCM using the Zeiss software (Carl Zeiss AxioVision software). The number of processes was counted in 10 different fields/*per* each group and then averaged using Fiji ImageJ software similar as Pemberton (Pemberton et al. 2018).

Statistical analysis

All the data are presented as mean ± standard deviation. Comparisons among multiple groups (negative control,

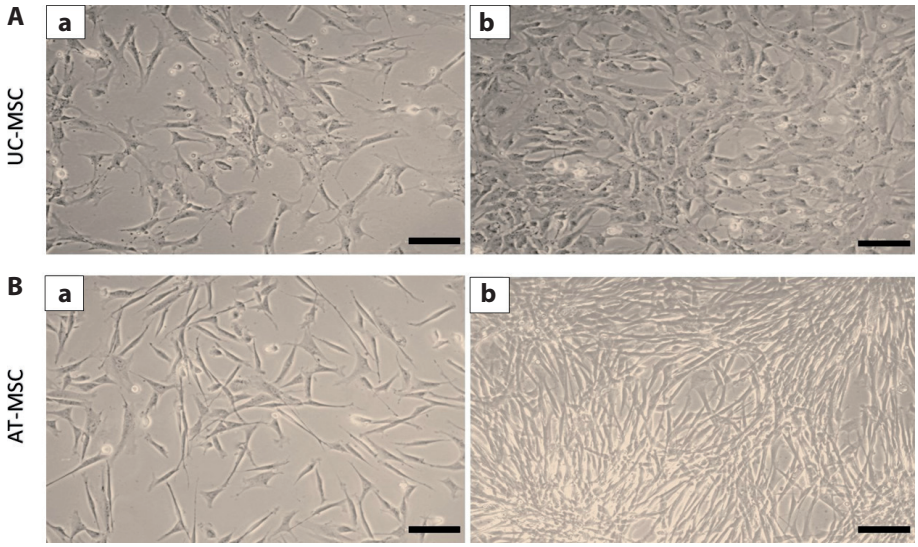

Figure 1. Morphological comparison of UC-MSC (umbilical cord mesenchymal stem cells; **A**) and AT-MSC (adipose tissue mesenchymal stem cells; **B**). UC-MSC: passage 3, day *in vitro* 6 (DIV6) (**a**); passage 3, DIV10 (**b**). AT-MSC: passage 3, DIV3 (**a**); passage 3, DIV7 (**b**). Scale bars: 50 µm.

UCMSC-CM and ATMSC-CM) were performed by One-way ANOVA for each day using GraphPad Prism 5.0 (GraphPad Software, Inc, San Diego, CA). Significance was set as follows: * $p < 0.05$, $p < 0.01$ and ** $p < 0.001$.

Results

Morphologic characteristics

Using above-mentioned protocols, we were able to isolate MSC from adipose tissue and umbilical cord. Morphological comparison of UC-MSC and AT-MSC showed some typical features for each population, while UC-MSC showed fibroblast-like shape (Fig. 1A), the AT-MSC revealed spindle-like shape (Fig. 1B).

Flow cytometry profile

Flow cytometry analyses of AT-MSC and UC-MSC from third passage showed that both cell populations expressed CD markers typical for MSC. AT-MSC expressed primarily CD29 (99.4%) and CD90 (78.6%) but low CD45 (1.3%), while UC-MSC from identical passage showed higher expression of CD45 (11.6%), CD29 (98.4%) and reduced CD90 (46.2%) in comparison with AT-MSC (Fig. 2).

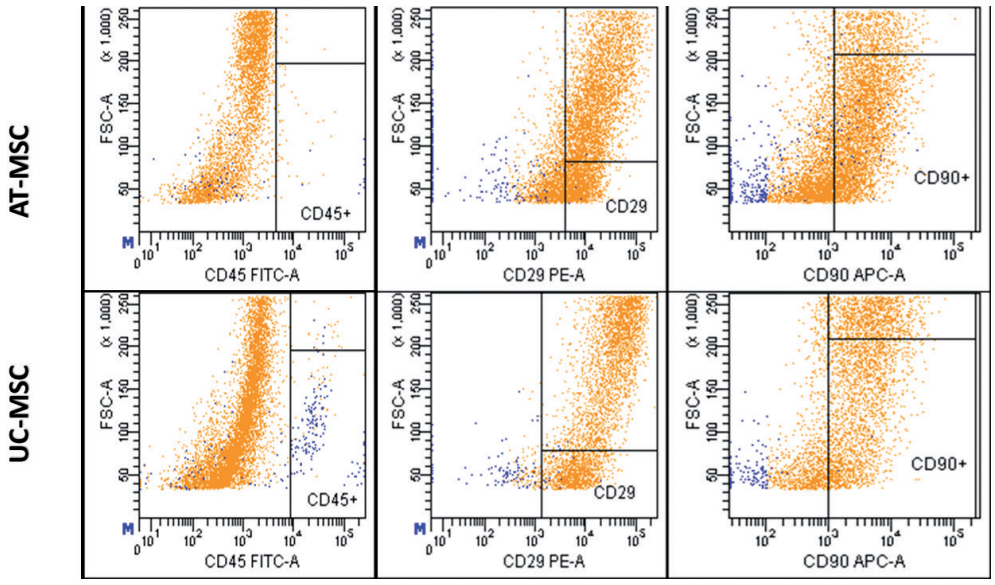

Figure 2. Representative flow cytometry analyses with canine CD markers of AT-MSC CD45 (1.3%), CD29 (99.4%) and CD90 (78.6%) (upper panel) and UC-MSC CD45 (11.6%), CD29 (98.4%) and CD90 (46.2%) (lower panel) from passage 3 presented as scatter blot.

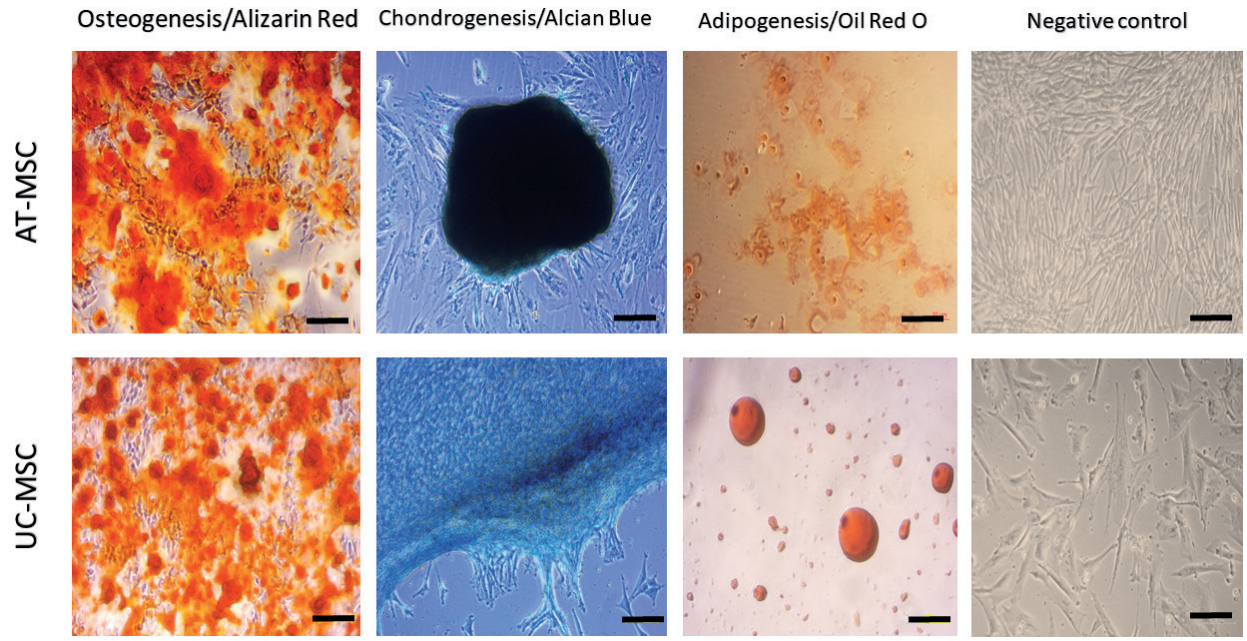

Figure 3. Multilineage differentiation of AT-MSC (upper panel) and UC-MSC (lower panel). Figures represent multilineage potential of canine MSC isolated from adipose tissue and umbilical cord, differentiated into osteocytes (Alizarin Red), chondrocytes (Alcian Blue) and adipocytes (Oil Red O). Scale bar 50 μm.

Multilineage potential

Three-lineage potential was detected by using commercial StemPro® Differentiation Kits. Present data confirmed that canine AT-MSC and UC-MSC after 21 days of incubation in specific differentiation medium underwent a high degree of biomineralizing osteogenesis. This was confirmed by Alizarin Red S which is an anthraquinone dye used to stain calcium deposites. Similarly, we captured a significant chondrogenic potential. In addition, chondrocytes migrating from spherical chondrocyte-like aggregates revealed intense Alcian Blue staining, which is typical for chondrogenesis. On the contrary, we found a low degree of adipogenesis, with limited vacuole formation and Oil Red staining (Fig. 3).

Immunohistochemistry characterization of NPCs culture

For NPCs characterization, we used NF200 and GFAP antibody. NF200 is widely accepted as marker for large myelinated A-β fibers of neurons, while GFAP is expressed by astrocytes and ependymal cells. Population of NPCs was

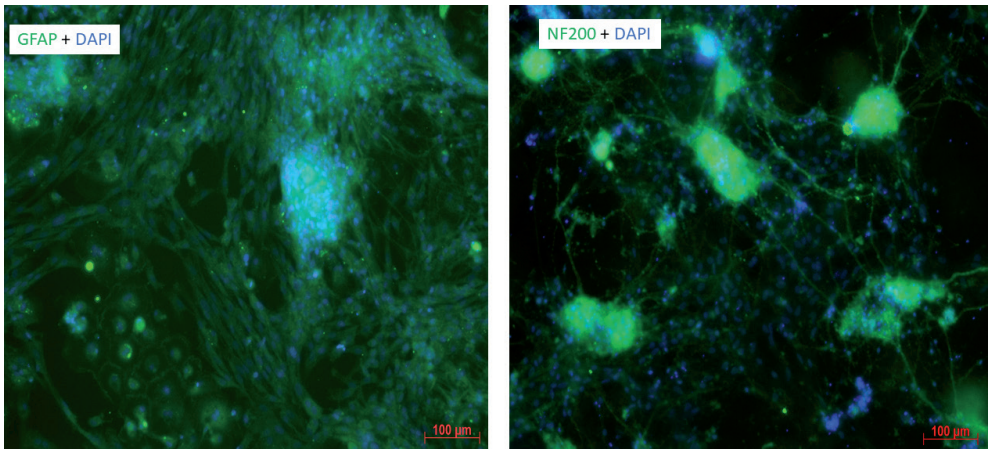

Figure 4. The expression of glial (GFAP) and neural (NF200) markers. The cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) detected by fluorescent microscopy. Scale bar 100 μm.

positive for both markers (Fig. 4). Furthermore, we observed formation of neurosphere-like structures of various diameters, which disintegrate spontaneously and formed connected networks of neural cells populations after further *in vitro* culture conditions.

Neurite outgrowth

Bradford measurement as quantitative method for analyses of conditioned media from both sources showed only mild differences between concentrations of proteins in UC-MSCCM and AT-MSCCM (1.40 mg/ml and 1.17 mg/ml proteins, respectively). Thus, both CM were diluted to final concentration of 1.0 mg/ml for neurite outgrowth experiment.

In vitro study confirmed neurotrophic-like stimulatory properties of UC-MSCCM and AT-MSCCM with promoted neurite outgrowth. Positive control medium, containing high concentration of neurotrophic factors, showed the most significant effect (Fig. 5) on the number of neurite processes from day 1 to day 3 (91.98% increase of number of processes compared to the negative control over 3 days of cultivation) (Fig. 6). However, the concentration of the neurotrophic factors in CM of positive control (Positive CTR) was significantly above the physiological range therefore, we did not compare these data with both experimental groups (UC-MSCCM and AT-MSCCM). Furthermore, UC-MSCCM showed a significant increase of number of processes compared to the negative control at day 2 ($p < 0.01$) and day 3 ($p < 0.01$) but not at day 1, although data were close from reaching significant results ($p = 0.0709$).

Similarly, AT-MSCCM showed a significant enhancement of processes outgrowth compared to the negative control at day 1 ($p < 0.05$) but not the following days (day 2: $p = 0.5689$; day 3: $p = 0.69$).

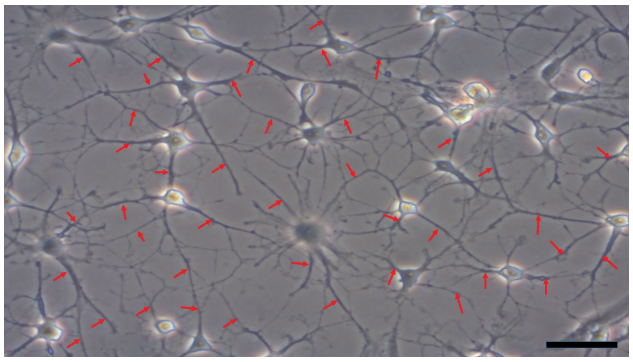

Figure 5. Analysis of neurite growth. The average number of processes was measured using Zeiss's software. An arrow (in red) was placed on each of the neurite in each field, excluding those that are not completely in the field. Scale bar 50 μm. (For color figure see online version of the manuscript).

Discussion

Several adult tissues have been identified as potential sources of MSC including bone marrow, adipose tissue, placenta, umbilical cord, umbilical cord blood, amnion, dental pulp, liver parenchyma and many others (Berebichez-Fridman and Montero-Olvera 2018; Humenik et al. 2019; Kholodenko et al. 2019). In order to isolate MSC from adipose tissue and umbilical cord, we used a combined method of mechanical disruption and enzymatic digestion with the enzyme collagenase I or IV (Buyl et al. 2015; Mastrolia et al. 2019). The enzymatic process lasted 25–45 min, depending on the amount and structure of the digested fraction. However, prolonged digestion could damage cells, because of cells sensibility to proteolytic enzymes (Mushahary et al. 2018; Mastrolia et al. 2019).

Isolated MSC revealed differences in several parameters, similarly as reported in previous experiments (Berebichez-Fridman and Montero-Olvera 2018). MSC derived from adipose tissue showed a spindle shape and 100–140 μm in perimeter. On the other hand, MSC isolated from umbilical cord showed fibroblast-like shape and cells were significantly smaller, ranging around 80 μm.

Interesting data were obtained while monitoring the multidifferentiation capacity of isolated cell populations. Cells from each population were tested for their capacity to differentiate into osteogenic, chondrogenic and adipogenic line. MSC isolated from both sources, adipose tissue and umbilical cord, showed a very good ability to differentiate into osteogenic and chondrogenic line, but very little or no ability to differentiate towards adipogenic cells, even after repeating the experiment several times. This inability of MSC adipogenesis has been described also in other studies (Kern et al. 2006; Kozlowska et al. 2019). A key role in this phenomenon may play up regulation of bioactive molecules such as: Runx2, Wnt10b, RhoA, which are capable of mediating osteogenesis but suppressing adipogenesis (Zhang et al. 2012).

Flow cytometry of CD surface markers expression outlined few variations as well. AT-MSC from the third passage showed low expression of CD45+ cells and high expression of the CD 29+ and CD90+ cells. On the contrary, UC-MSC from the same passage showed higher expression of CD45+ and less expression of CD90+ cells while slight difference was detected for CD29+. From flow cytometry results, we can conclude that each cell population is unique and homogeneity can be achieved by passaging or modifying isolation technique. It should be noted that the actual expression of positive and negative markers also depends on the source from which the MSC were isolated (Maleki et al. 2014) as well as on the age of donors (Lin et al. 2013) and the cellular aging (replicative capabilities) (Yang et al. 2018). In addition, slight differences may occure even between individual samples.

It is well documented that MSC are stimulating the environment and other cells through the paracrine activ-

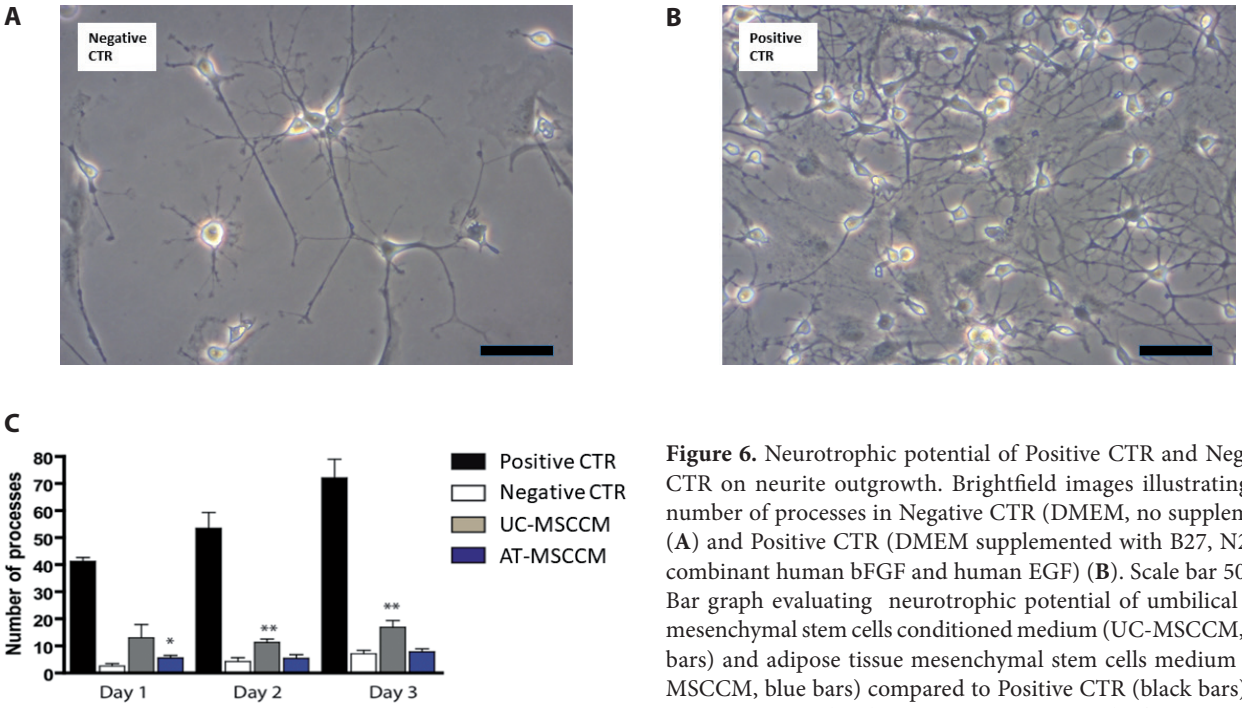

Figure 6. Neurotrophic potential of Positive CTR and Negative CTR on neurite outgrowth. Brightfield images illustrating the number of processes in Negative CTR (DMEM, no supplement) (**A**) and Positive CTR (DMEM supplemented with B27, N2, recombinant human bFGF and human EGF) (**B**). Scale bar 50 μm. Bar graph evaluating neurotrophic potential of umbilical cord mesenchymal stem cells conditioned medium (UC-MSCCM, grey bars) and adipose tissue mesenchymal stem cells medium (AT-MSCCM, blue bars) compared to Positive CTR (black bars) and Negative CTR (white bars) on neurite outgrowth of NPCs primary culture during 3 days of cultivation (**C**). Data represent mean value ± SEM. Unpaired t-test between the UC-MSCCM or AT-MSCCM and negative control conditions within the same day * $p < 0.05$ and ** $p < 0.01$.

ity *via* production of growth factors, mediators and other bioactive molecules, included in a conditioned medium (Humenik et al. 2019; Rezaie et al. 2019; Maacha et al. 2020). Previous study focused on proteomic analyses of MSCCM from adipose tissue and umbilical cord showed, that there are differences in composition of secretomes from these sources (Shin et al. 2021). However, according to previous studies, the gender or strain differences have no major effect on composition of CM (Barzilay et al. 2009; Teshima et al. 2019). Here, we have compared neurotrophic stimulation of two CM derived from MSC of different origin. Our findings showed that both UC-MSCCM and AT-MSCCM enhanced neurite growth. Interestingly, this effect was more pronounced with UC-MSCCM than AT-MSCCM. The accelerated neurite outgrowth can be explained by the paracrine potential of MSC mediated through increased production of HGF (hepatocyte growth factor), VEGF (vascular endothelial growth factor), IGF (insuline growth factor), EGF (epidermal growth factor), GDNF (glial cell-line derived neurotrophic factor), BDNF (brain-derived neurotrophic factor), NT3 (neurotrophin-3), TGF (transforming growth factor), angiopoietin, and adhesion molecules (Pawitan 2014; Dabrowski et al. 2017; Dong et al. 2018; Mukai et al. 2018; Cofano et al. 2019; Xiang et al. 2020).

These data are in line with present findings revealing neurite outgrowth, which was most likely promoted by UC-MSC-secreted BDNF and GDNF (Qi et al. 2018). Furthermore, *in vitro* co-cultures showed that UC-MSC promote neuronal/glial survival and neuritogenesis also through the secretion of BDNF and β-NGF (Pawitan et al. 2017). Characterization of UC-MSCCM by antibody-based protein array analyses and by enzyme-linked immunosorbent assays (ELISA) showed enrichment of IGF-1, HGF, VEGF and TGF-β (Arutyunyan et al. 2016, Ahangar et al. 2020). In addition, neuroprotective efficacy of UC-MSC and its products was confirmed also *in vivo* by treating cerebral palsy (Wang et al. 2015; Okur et al. 2018) or SCI (Cheng et al. 2014).

In summary, present study provides evidence that UC-MSCCM has a greater effect on neurite outgrowth compared to AT-MSCCM, suggesting that UC-MSC are probably secreting more neurotrophic factors (Cofano et al. 2019). Indeed, the number of processes counted are not the only indicator of neurite growth, but also length or ramification are key parameters for neuritogenesis that need to be taken in account in the future studies.

Conclusion

In this study, we showed that CM derived from MSC of different tissue origin have a beneficial effect on neurite outgrowth. Interestingly, we found that UC-MSCCM had

a more stimulatory effect on neurite outgrowth when compared to AT-MSCCM, which may be associated with the capacity of releasing a higher content of neurotrophic factors. These data correlate with previous study of UC-MSCCM revealing higher secretion of neurotrophic factors (bFGF, NGF, NT3, NT4, GDNF), lower immunogenicity in the host tissue, higher anti-inflammatory effect in injured nervous tissue than by AT-MSCCM (Cofano et al. 2019).

Acknowlegment. This study was supported by grants APVV-19-0193, VEGA 1/0376/20.

Authors contribution. DC, FH and SJ conceived of the presented idea. FH, SJ, MM, MC, and NH planned the experiments. MZ, LH, AV-A were crucial for obtaining tissues for MSC isolation. FH, ZV and SJ aided with cell isolation, cultivation and passaging. FH, DM performed the MSC and CM characterization. MZ, SJ, MM and FH carried out the experiments on NPC. DC, FH, SJ contributed to the interpretation of the results. All authors wrote and revised the manuscript. DC finantially supported the experiments.

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Received: May 25, 2021
Final version accepted: June 30, 2021