Neural differentiation of canine mesenchymal stem cells/multipotent mesenchymal stromal cells

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Abstract

Background: Ability of adipose tissue-derived multipotent mesenchymal stromal cells/mesenchymal stem cells (ASCs) to differentiate in neural lineages promises progress in the field of regenerative medicine especially for replacing damaged neuronal tissue in different neurological disorders. Reprogramming of ASCs can be induced by supplying growth medium with chemical neurogenic inductors and/or specific growth factors. We investigated the neural differentiation potential of canine ASCs using several growth media (KEM, NIMa, NIMb, NIMc) containing various combinations of neurogenic inductors: B27 supplement, valproic acid, forskolin, N2-supplement and retinoic acid. First the cells were preconditioned in proliferation medium, followed by induction of neuronal differentiation. Six canine ASCs cell lines were assessed, half from female and half from male donors. The cell morphology, growth dynamics, viability were observed along with expression of neuron and astrocyte specific markers, which were assessed by immunocytochemistry and flow cytometry.

Results: After 3, 6 and 9 days, elongated neural-like cells with bipolar elongations were observed and some oval cells with light nuclei appeared. After three and nine days of neural induction, differentiation into neurons and glial cells was observed. Expression of neuronal markers tubulin beta III (TUBB3), neurofilament H (NF-H) and glial fibrillary acidic protein (GFAP) was observed by immunocytochemistry. High GFAP expression (between 70 and 90% of all cells) was detected after three days of growth in neural induction medium a (NIMa) by flow cytometry, and expression of adult neuronal markers NF-H and microtubule associated protein-2 (MAP2) was detected in around 25% of cells. After nine days of ASCs differentiation a drop in expression rates of all markers was detected.

There were no differences between neural differentiation of ASCs isolated from female or male dogs.

Conclusions: The differentiation repertoire of canine ASCs extends beyond mesodermal lineages. Using a defined neural induction medium the canine ASCs were able to transform to neural lineages, bearing markers of neuronal and glial cells and also displayed the typical neuronal morphology. Differentiated ASCs can be a source of neural cellular lineages for regenerative therapy of nerve damage and also could be applicable for modeling of neurodegenerative diseases.
Multipotent mesenchymal stromal cells (MSCs), also commonly referred to as mesenchymal stem cells, are self-renewing, multipotent, adult stem cells that have a mesodermal and neuroectodermal origin (1,2). They are found in many tissues, such as adipose tissue, bone marrow, cord blood, chorionic folds of the placenta, amniotic fluid, blood, lungs, etc., most of which are easily accessible and represent a potentially important source of cells. The ability of MSCs to transdifferentiate into osteoblasts, chondroblasts and adipocytes in vitro conditions has been demonstrated in several studies (3,4). Until the year 2000, there was a widely accepted hypothesis that MSCs are capable to differentiate only into the mesodermal tissues. However, this was challenged when rat MSCs isolated from the bone marrow exposed to butyl hydroxyanisole, β-mercaptoethanol and dimethylsulfoxide expressed proteins specific for the nervous system (5).

Most studies on neural differentiation of MSCs were carried out with human and rodent cells (2,5–13). In veterinary medicine, dogs are interesting for development of novel regenerative treatments, and in addition to benefiting canine patients, these therapies might show translational potential as dogs could be very interesting model of human neurological disorders. Few studies already reported the induction of canine MSCs into neural lineages (14–17). GFAP, MAP2, A2B5, S100, TUBB3, nestin and NEUN are markers of neuronal cellular faith. Canine adipose tissue-derived multipotent mesenchymal stromal cells (ASCs) could be induced to express some typical neuronal genes after growth in the presence of neurogenic inductors valproic acid and forskolin (14,16), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (15), in commercial neurogenic differentiation medium (18) and in medium containing N2 supplement, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (19). Neurospheres were used to enrich for neural lineages expanded enough to transplant (19) and neurospheres generated from canine ASCs grown in hypoxia showed higher expression of neuronal marker nestin (20). Recently neural differentiation of human ASCs was also evoked by conditioned medium obtained from glial cells (21). Functional recovery following spinal cord injury in a dog was reported by transplantation of neurally differentiated ASCs (16). On the other hand, pre-differentiation of ASCs was not a prerequisite step for better functional recovery of rat spinal cord injury (22) and the same condition was successfully treated in dogs through allogenic
ASCs transplantations (23).

In the present study four different cell growth media were tested to determine their ability to induce differentiation of canine ASCs into neural lineages. The media components included B27 supplement, valproic acid, forskolin, N2-supplement and retinoic acid. One of the tested media enabled prominent neural differentiation, observed by morphological changes at the cellular level, as well as by expression of glial marker GFAP and neuronal markers NF-H and MAP2, latter detected by immunocytochemical stainings and flow cytometry.

Methods

Cell origin

Adipose tissue was collected by veterinarians at veterinary clinics. All dogs were privately owned patients and were under general anesthesia for other procedures, not purposefully to collect samples for this study and none of the dogs was euthanised. All dogs’ owners gave their written consent to use the removed adipose tissue for research purposes and all the experiments were performed according to the procedures and guidelines approved by the National Health Service branch of the Slovenian Ministry of Health. Additional ethical permission was not needed according to Slovenian legislation and interpretation by Administration for safe food, veterinary and plant protection, which is responsible for issuing licenses for experiments with animals. Subcutaneous adipose tissue (<1 cm3) was aseptically removed from the back area between the dog’s scapulae. Samples were obtained from six different dogs aged 2 to 9 years (mean age 5.5 years) of different breeds and both sexes, three males and three females.

Isolation of canine ASCs

Canine ASCs were isolated from adipose tissue and characterized based on the routine protocols developed by the Animacel Ltd. Briefly, fat tissue was minced, washed three times in PBS buffer and digested over night with an equivalent amount of collagenase I solution containing 2 mg/ml collagenase I and 4 mg/ml BSA in HEPES buffer, pH 7.4. Collagenase I activity was stopped by a double volume of PBS and the resulting cell suspension was filtered through a falcon strainer (pore
size 100 µm). After centrifugation cells were re-suspended in DMEM Glutamax (Gibco) medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and plated at a density of 10^5 cells/cm². After 24h non-adherent cells were washed off. ASCs were cultured until 80% confluence was reached, detached, counted with Bürker Turk Chamber hemocytometer and passaged. Again, cells were maintained until 80% confluence, detached and cryopreserved in a freezing medium consisting of DMEM Glutamax, FBS and DMSO for later use.

Cultivation of ASCs

The high cell density is a key parameter to the successful differentiation of multipotent mesenchymal stromal cells into neural lineages. Isolated ASCs (first passage) were plated into different growth flasks depending on the designed experiment. During the optimization and monitoring of the morphology of undifferentiated and differentiated cells, two T25 flasks were used for cells from each dog, plated at a density of 10^6 cells/cm². Three additional T25 flasks were required for flow cytometry, for each point of differentiation and for each animal, with the cells at the same density. Furthermore, for the immunocytochemical characterization of differentiated ASCs, individual round dishes with diameter of 35 mm were used with cells at a density of 8x10^4 cells/cm² and 24-well plates with cells plated on glass cover slips coated with laminin at a density of 2x10^4 cells/cm².

Neural differentiation

Canine ASCs in a second passage were plated in different flasks based on the type of the experiment. Cells were grown in cultivation medium, which was previously demonstrated to be specific for canine ASCs cultivation (30) and was developed by Animacel Ltd. After 24 hours, the cultivation medium was replaced by the pre-differentiation neural induction medium (mitogenically stimulated; STIM). Two pre-differentiation media were tested. STIM1 consisting of DMEM Glutamax, EGF (20 µg/ml), bFGF (20 µg/ml) and 1x B27 supplement; and STIM2 consisting of DMEM Glutamax, β-mercaptoethanol (100 µM) and 20% FBS. After 24 hours, the pre-differentiation medium was replaced by the neural
differentiation medium. Four different differentiation media were prepared:

1. **KEM** media consisting of DMEM Glutamax, 1% FBS, 2x B27 supplement, valproic acid (2mM), forskolin (10 µM), hydrocortisone (1 µM), KCl (5 mM), butylated hydroxyanisole (BHA; 200 µM), insulin (5 µg/ml) and 0.1% penicillin/streptomycin.

2. **NIMa** (neural induction medium a) composed of DMEM Glutamax, 2x B27 supplement, 1% N2-supplement, 1% FBS, 0.1% penicillin/streptomycin and 10 nM retinoic acid.

3. **NIMb** consisting of DMEM Glutamax, 2x B27 supplement, 1% N2-supplement, 1% FBS, 0.1% penicillin/streptomycin and 100 nM retinoic acid.

4. **NIMc** composed of DMEM Glutamax, 2x B27 supplement, 1% N2-supplement, 1% FBS, 0.1% penicillin/streptomycin and 10 µM retinoic acid.

The cells were further processed for morphological, immunocytochemical and flow cytometry analysis after 3, 6 and 9 days following neural induction.

**Viability assay**

To determine the viability of canine ASCs cells after exposure to neural differential medium, dye exclusion assay was used. At daily time points from 0 to 9 days after induction of differentiation viability was determined by adding Trypan Blue dye into cell suspension and counting live/dead cells using a Bürker Turk Chamber under the light microscope. The viability of cells grown in control media was determined for comparison to neurally differentiated cells. Differences in cell viability between control and differentiated ASCs cells were calculated and statistical significance determined using Student’s t test.

**Immunohistochemistry on dog brain**

Dog brain tissue sections were used as control for the reactivity of antibodies and was obtained at Veterinary faculty of University of Oslo. The dog was three years old and euthanized due to internal organ failure. The brain was surgically removed, cut in small cubes and fixed in 4% paraformaldehyde.
at 4 °C. Afterwards, pieces of brain were washed twice with PBS and incubated in 30% sucrose at 4 °C overnight. Pieces were then washed in PBS and transferred in molds with O.C.T. medium, dipped in liquid nitrogen and stored at -20 °C. Next, 14 µm cryosections were cut with cryotome (Leica), followed by immunohistochemical stainings. First, blocking of unspecific epitopes was performed in 10% FBS, 1% milk powder, 0.02% Na-azide in TBST (pH 7,2) for 30 minutes at room temperature. Sections were then incubated overnight at 4 °C in the dark with the following primary antibodies: rabbit anti-GFAP (1:500, G3893, Sigma) conjugated with Alexa Flour 488 (Life Technologies, USA); mouse anti-NF-H (1:400, AB1989, Millipore), chicken anti-bIII tubulin (1:400, AB9354, Chemicon), rabbit anti-MAP2 (1:400, AB5622, Millipore) and chicken anti-nestin (1:200, ABIN187958, Neuromics Antibodies). The next day slides were washed five times with PBS for 5 min and once with TBST and incubated with secondary donkey anti-rabbit Cy2 conjugated IgG antibody or goat anti-mouse Cy3 conjugated IgG antibody (both 1:500, Jackson Immunoresearch) for 2h at room temperature in the dark. Anti-GFAP antibody was previously labelled with mouse anti-rabbit Alexa Flour 488 IgG using Alexa Fluor® 488 Protein Labeling Kit (Molecular Probes). The nuclei were counterstained with Hoechst (1:1000, Molecular Probes). Slides were then washed with PBS twice for 5 minutes and mounted in glycerol. Stainings were visualized with a confocal microscope (Zeiss LSM 710).

**Immunofluorescence analysis of cells**

Immunocytochemistry was performed on cells to evaluate the presence of the neural markers after the induction with differentiation medium. One 24-well plate was prepared per each time point with small glass cover slips coated with laminin (10 µg/ml). Next day, the second passage canine ASCs were plated at a density of 2x104 cells/cm2 in 1 ml of the basal growth medium (formulation property of Animacel Ltd.). At three time points, pre-differentiation, 3 and 9 days of differentiation, cells were fixed with 4% paraformaldehyde (pH 7.4) for 10 min at room temperature, rinsed three times in PBS and permeabilized with TBST for 5 min. Blocking of unspecific epitopes was performed with blocking solution (10% FBS, 1% milk powder, 0.02% Na-azide, TBST, pH 7,2) for 1h. Cells were then incubated overnight at 4 °C with the following primary antibodies: rabbit anti-GFAP (1:400, G3893, Sigma)
conjugated with Alexa Flour 488, rabbit-anti-NF-H (1:400, AB1989, Millipore), rabbit anti-MAP2 (1:200, AB5622, Millipore). The next day cells were rinsed three times with PBS and one time with 2% FBS in PBS and incubated with secondary donkey anti-rabbit Cy2 conjugated IgG antibody or secondary donkey anti-mouse Cy3 conjugated IgG antibody (both 1:500; Jackson Immunoresearch) for 2h at room temperature in dark. GFAP antibody was previously labelled as described above. The nuclei were counterstained with DAPI. The same protocol was applied to undifferentiated and differentiated ASCs. Stainings were visualized with a confocal microscope (Zeiss LSM 710) or fluorescent microscope (Nikon Eclipse 80i). Neural differentiation was detected by observing random viewing fields under the microscope and by comparing cellular morphology. NF-H and MAP2 positive cells were identified as neuronal lineage and GFAP-positive cells were identified as glial lineage (astrocytes).

**Flow cytometry**

At three time points after neural differentiation (pre-differentiation, 3 and 9 days of differentiation) of ASCs the cell cultures were detached for flow cytometry analyses. Confluent cells were harvested using trypsin (TrypLE™ Express, Gibco) digestion for 5 min at 37 °C, centrifuged for 5 min at 1400 rpm at 4 °C and resuspended at a concentration of 10^7 cells/ml in PBS. Five aliquots were prepared per time point (pre-differentiation, 3 and 9 days of differentiation) per animal each containing 100 µl (concentration of 10^6 cells/ml) and were transferred to flow cytometry facility at Medical Faculty, University of Ljubljana. The aliquots were centrifuged for 5 min at 1600 rpm at room temperature and pellet was fixed with 4% paraformaldehyde (pH 7.4) for 10 min at room temperature, rinsed three times in PBS, and in parallel permeabilized with 0.1% Triton X-100, and blocked with 2% FBS, 5% milk powder, 0.02% penicillin/streptomycin for 10 min. Cells were incubated for 30 min in the dark at 37 °C with following primary antibodies: rabbit anti-GFAP (1:400, G3893, Sigma) conjugated with Alexa Flour 488 (Life Technologies), rabbit anti-NF-H (1:400, AB1989, Millipore), rabbit anti-MAP2 (1:200, AB5622, Millipore). After incubation samples were centrifuged 5 min at 1600 rpm, rinsed two times with 2% FBS in PBS and incubated with secondary donkey anti-rabbit IgG Cy2 conjugated antibody (1:500,
Jackson Immunoresearch). Anti-GFAP antibody was previously conjugated with Alexa Flour 488 (as described above). All samples were washed in PBS and stored at RT prior to analysis using a BD FACS Canto flow cytometer (BD Biosciences). Appropriate isotype matched controls conjugated to FITC were used to identify nonspecific staining for GFAP antibody. For unconjugated primary antibodies, controls included isotype matched unconjugated primary controls and incubation with the secondary antibody alone. Data was analyzed using FACSDiva™ version 6.1.2 (BD Biosciences) analysis software.

**Statistical analysis**

Immunocytochemical staining and cell morphology were evaluated only qualitatively by observing random viewing fields by observer blind to the differentiation conditions. Differences in cell numbers and cell count between control and differentiated ASCs were analyzed by Student’s t test, with $P<0.05$ considered as statistically significant. Differences between groups in expression of neuronal markers detected by flow cytometry were analyzed by ANOVA followed by Student’s t test, with $P<0.05$ considered as statistically significant.

**Results**

**Morphology**

Morphological characterization of ASCs was assessed at 6 time points: untreated cells at 80% confluence, 24 and 48 hours after addition of pre-differentiation medium and 3, 6 and 9 days after induction of differentiation of ASCs. Canine ASCs cultured in basal growth medium displayed typical fibroblast-like morphology. First the cellular growth in pre-differentiation media STIM1 and STIM2 was assessed. STIM2 turned out to be highly toxic to the cells but STIM1 was suitable for cultivation. STIM1 contained L-glutamine, B27 supplement and two growth factors, namely EGF and bFGF. The pre-differentiation was introduced in order to enhance ASCs proliferation (by EGF and bFGF) and to slowly direct the lineage commitment to neural cell types and maintain the neural cells, the latter by B27 supplement, which includes a retinoic acid derivative. The first morphological changes were noticed after 24 hours of treating ASCs with the pre-differentiation medium STIM1 when neuronal-like cells
appeared, identified by their elongated shape with bipolar elongation. Additionally, some oval cells appeared with light nuclei (Figure 1A). The same cellular morphology was noticed after 48 hours of growth in STIM1 medium, with more dead cells detected (Figure 1B). ASCs seeding density affected the efficiency of differentiation, which suggests that the cell communication is essential for inducing the ASC to form neural phenotypes.

Further, growth in four differentiation media was assessed. KEM medium was highly toxic to the cells, which died already after 3 days in this medium (Figure 2). Its toxic effects were probably due to the abundance of neural differentiation inducing factors and therefore we further tested three additional differentiation media with different compositions.

NIMa, NIMb and NIMc all contained L-glutamine, B27 and N2 supplements and varying concentrations of retinoic acid. NIMa, NIMb and NIMc were more suitable for cell culturing and neuronal differentiation (Figure 3). During growth in NIM differentiation media, the number of cells with neuronal phenotype increased and they appeared more branched. Already at 3 days after the onset of differentiation, some cells obtained a neuron- or glial-like morphology with arterial tree-like processes (Figure 3A, D and G). The concentration of retinoic acid in NIMa, NIMb and NIMc media was 10nM, 100nM and 10µM. The most prominent neural like morphologic changes were observed after 6 days cultivation in NIMa medium (Figure 3B), although they were also observed in the other two media. The concentration of retinoic acid in NIMb and NIMc might have been too high, since many apoptotic cells were also observed. Therefore, NIMa medium was determined to be the most suitable neural induction medium and all subsequent experiments were conducted in NIMa medium. Following 9 days incubation in NIMa medium neural-like cells appeared more elongated and branched, their cytoplasmic elongations resembling dendrites (Figure 3C).

**Viability**

Cellular numbers were determined at three time points. $10^5$ cells were seeded per ml of growth medium then the cells were counted after 24-hour pre-differentiation in STIM1 medium and at 3 and 9
days after induction of differentiation by NIMa medium (Figure 4A). Six lines of canine ASCs were used, three from female and three from male donors. No differences in growth dynamics between cells derived from different sexes were observed. The individual growth curves followed the same trends, reaching the peaks in cellular numbers at three days after the differentiation commenced (Figure 4A). The cellular viability dropped at day 9 of differentiation, this drop was statistically significant (p = 0,006) and was lower for ASCs derived from female donors than from male dogs, i.e. 83,4 ± 1,0% vs. 93,5 ± 2,3% (Figure 4B).

**Immunofluorescence**

Canine brain cryosections were stained with antibodies directed against neural lineages to determine the reactivity of these antibodies against canine epitopes and thus the usefulness for their further use on cellular cultures. Ten different antibodies were tested, which were directed against neurofilament (NF-H), TUBB3, NES (nestin), MAP2 and GFAP. Astrocytes were marked by antibodies directed against GFAP and neurons by anti NF-H, MAP2 and TUBB3 (Figure 5), antibodies directed against nestin did not stain nestin in the canine brain.

Differentiation of canine ASCs into neural lineages was determined by expression of TUBB3, GFAP and NF-H. Immunocytofluorescence analysis revealed the presence of neuronal cytoskeleton proteins TUBB3 and NF-H and GFAP, a marker of glia cells, after 3 and 9 days of growth in differentiation medium NIMa (Figure 6 and Figure 7).

**Flow cytometry**

Expression of neural markers GFAP, MAP2 and NF-H in ASCs grown in differentiation media for 3 and 9 days was further characterized by flow cytometry. As the size of multipotent mesenchymal stromal cells is similar to the size of lymphocytes the gating of cells was done similarly. The expression of markers was comparable for differentiated cells derived from female and male dogs (Figure 8). Average expression of GFAP in undifferentiated cells from male donors was 2,3 ± 1,4%, MAP2 0,7 ± 0,4 % and NF-H 1,4 ± 0,8% and from female donors GFAP 5,3 ± 1,1 %, MAP2 1,4 %, NF-H 0,9 ± 0,3 %.
After 3 days of differentiation in NIMa media expression in cells derived from male dogs was: GFAP 90.4 ± 0.6 %, MAP2 33.6 ± 0.1 %, NF-H 32.6 ± 0.8 % and in ASCs from female dogs: GFAP 73.5 ± 22.3 %, MAP2 22.8 ± 5.4 %, NF-H 33.3 ± 1.4 %. The expression of all markers dropped 6 days later (after 9 days of differentiation) although less in cultures of male derived ASCs (GFAP 17.8 ± 12.1 %, MAP2 9.0 ± 5 %, NF-H 8.7 ± 0.4 %) than female dogs derived ASCs cultures (GFAP 30.9 ± 14.3 %, MAP2 13.6 ± 10.7 %, NF-H 11.7 ± 11.0 %) (Figure 8).

Discussion

The potential of differentiation of MSCs into neural cells makes them interesting and potentially useful for neural reconstitution in neurodegenerative diseases, stroke and spinal cord injuries. Whether the transplantation of ASCs alone would be sufficient to treat spinal cord injuries and other neurological disorders, or the in vitro differentiation would be needed prior to transplantation, is still being studied. Here we confirm that canine adipose tissue derived MSCs are capable of neural differentiation in vitro and explore which kind of neural induction media is the most suitable for neural differentiation of canine ASC. In previous studies, rat and human multipotent mesenchymal stromal cells were shown to transdifferentiate into neural phenotypes by exposing the cells to a variety of neurogenic inductors, such as β-mercaptoethanol, butylated hydroxyanisole, potassium chloride (KCl), valproic acid and forskolin (2,5,8,11,12). Alternative methods of inducing chemical differentiation to a neural lineage involved the addition of growth factors such as bFGF, EGF, neuroblast factor (N2), B27 supplement and retinoic acid (7,13,15,23). One study also showed that canine adipose tissue derived stromal cells could be differentiated into neuronal cells by incubation with dibutryl cyclic adenosine monophosphate (dbcAMP) and isobuthylmethylxanthine (IBMX) (17).

We tested two pre-differentiation media to condition the cells to neural differentiation and serum-free medium (STIM1) with added growth factors EGF, bFGF and B27 supplement was suitable, whereas STIM2 turned out to be highly toxic to the cells, probably due to high concentration of β-mercaptoethanol. This pre-differentiation step was introduced due to previous studies showing that culturing ASCs under active proliferation conditions greatly improves their propensity to differentiate
toward neurogenic lineages (24). The neurogenic inductors in differentiation media tested were B27 supplement, valproic acid, forskolin, KCl and butylated hydroxyanisole (BHA) in KEM medium and B27 supplement, N2 supplement and retinoic acid in NIM media. As KEM was toxic to the cells and they died after only 3 days of incubation, the NIM media were further assessed for evoking the neural phenotype of canine ASCs. In NIMA medium, containing the lowest concentration of retinoic acid (10 nM), most prominent neural-like cellular phenotypes were noticed, thus this was the medium of choice for subsequent experiments. Retinoic acid, a metabolite of vitamin A, has roles in cell differentiation, neurite outgrowth and cell survival (25,26). It induces post-mitotic, neuronal phenotypes in various cells \textit{in vitro} (25,27,28) and might have been the crucial co-factor in NIMA medium for canine ASCs to switch from proliferation to differentiation. However, retinoic acid could be also highly toxic to cells both \textit{in vitro} and \textit{in vivo}, and higher concentrations of retinoic acid in media NIMb and NIMc was probably causing dying of cells. Although ASCs also seem to differentiate into neural phenotypes in media NIMb and NIMc, there were many apoptotic cells in cell culture with these two media.

Cell numbers were highest the third day after the addition of NIMA medium and no sex specific differences were observed in growth dynamics. Cells from male and female dogs grew similarly during the first 3 days of neural induction. The viability dropped at day 9 of differentiation and was lower for ASCs derived from female than male donors. This drop in viability was statistically significant between sexes. Stressors such as neurogenic factors present in NIMA medium and/or low serum concentration and poor nutrition might have increased ASCs death rate. Why female cells were more sensitive to these effects remain unexplained and will need to be studied in the future.

Cell population obtained by the cultivation of ASCs in neural-differentiation medium is often a mix of cells expressing one or more neural-specific markers. The most studied markers included nestin, TUBB3, S100 and GFAP (29). Differentiated rat and human multipotent mesenchymal stromal cells were shown to express mature neural markers such as GFAP, MAP2, TUBB3, neuron specific enolase
(NSE), possess voltage-gated calcium channels and the ability to upregulate the glutamate receptor (2,5,8,11,12). In the present study, after 24-hour incubation of canine ASCs in pre-differentiation medium STIM1 and 3 or 9 days incubation in NIMa differentiation medium cells expressed proteins characteristic for mature neurons and astrocytes. Immunofluorescence analysis showed expression of glial marker GFAP and neuronal markers TUBB3 and NF-H. The neural phenotype was confirmed by flow cytometry showing very high expression of GFAP after 3 days of growth in differentiation medium and increase of mature neuronal markers MAP2 and NF-H expressions in comparison to undifferentiated cells. In undifferentiated cells a basal expression of neural markers was already detected but at very low levels. This basal level of expression of neuronal markers in undifferentiated canine ASCs was already reported in a recent study, where some of the neuronal markers and neurotrophic factors were expressed already in undifferentiated cells (14). However, at the present it is not known whether some ASCs also express these neuronal markers, or perhaps during isolation from the adipose tissue some neuronal cells are being collected and cultured together with ASCs. After 9 days of growth in differentiation medium the expression of all markers tested dropped, which might have been due to de-differentiation of cells or apoptosis. To our best knowledge previous studies on ASCs neural differentiation did not follow the expression of neural markers in in vitro culture for more than 3 days, thus it remains unknown if the drop in the expression might have occurred in others studies if cells would remain in induction media for longer periods. However, this does suggest that for potential future use in regenerative medicine, the timing of neuronal differentiation would have to be explored meticulously, as it could be one of the critical points to achieve the highest quality of cells used for treatments.

Conclusions
The differentiation repertoire of canine ASCs clearly extends beyond mesodermal lineages. Their in vitro induction to form neural lineages, although partial, might be enough in the future to treat neurological conditions as a combinatorial treatment as MSCs already possess neuroprotective properties, such as anti-inflammation, anti-astrogliosis, neuronal extension and neuronal regeneration effects. However, as results of this study show, there are important differences in the ability of
different media to induce neural differentiation, therefore, in the future studies, the optimal composition of growth media will have to be determined, optimized and synchronized to develop general guidelines for neural induction of canine ASCc into neural cells.

Declarations

Ethics approval and consent to participate

Since study was conducted on client-owned animals undergoing routine clinical procedure with owner’s approval, no approval of ethical committee was needed according to Slovenian legislation and official opinion from The Administration of Republic of Slovenia for Food Safety, Veterinary and Plant protection, responsible for issuing ethical permits for animal experiments. The dog brain obtained at Veterinary faculty of University of Oslo, was from a dog, which was euthanized with pentobarbital due to internal organ failure.

Consent for publication

'Not applicable'

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Abbreviations

ASCs: Adipose tissue-derived multipotent mesenchymal stromal cells/mesenchymal stem cells; BDNF: Brain-derived neurotrophic factor; bFGF: Basic fibroblast growth factor; EGF: Epidermal growth factor; FBS: Fetal bovine serum; GFAP: Glial fibrillary acidic protein; MAP2: Microtubule associated protein 2; MSCs: Multipotent mesenchymal stromal cells; NGF: Nerve growth factor; NIMA: Neural induction medium a; NIMb: Neural induction medium b; NIMc: Neural induction medium c; NF-H: Neurofilament H; TUBB3: Tubulin beta III.

Competing interests

G.M. is partial owner of Animacel Ltd., L.M. is the CEO of Animacel Ltd.. Other authors have no competing interests to declare.

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Authors' contributions

V.K.G. and G.M. designed the study. V.K.G., L.M., and A.N.K. performed the experiments. V.K.G. and S.P.M. analysed the data and prepared Figures. S.P.M. and G.M. wrote the manuscript. All authors read and approved the final manuscript.

References

1. Ferroni L, Gardin C, Tocco I, Epis R, Casadei A, Vindigni V, et al. Potential for neural differentiation of mesenchymal stem cells. Adv Biochem Eng Biotechnol. 2013;129:89–115.

2. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001 Apr;7(2):211–28.

3. Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM. Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol. 2001 Oct;189(1):54–63.

4. Hauner H, Schmid P, Pfeiffer EF. Glucocorticoids and insulin promote the differentiation of human adipocyte precursor cells into fat cells. J Clin Endocrinol Metab. 1987 Apr;64(4):832–5.

5. Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res. 2000 Aug 15;61(4):364–70.

6. Ahmadi N, Razavi S, Kazemi M, Oryan S. Stability of neural differentiation in human adipose derived stem cells by two induction protocols. Tissue Cell. 2012 Apr;44(2):87–94.

7. Anghileri E, Marconi S, Pignatelli A, Cifelli P, Galié M, Sbarbati A, et al. Neuronal differentiation potential of human adipose-derived mesenchymal stem cells. Stem
8. Ashjian PH, Elbarbary AS, Edmonds B, DeUgarte D, Zhu M, Zuk PA, et al. In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. Plast Reconstr Surg. 2003 May;111(6):1922–31.

9. Franco Lambert AP, Fraga Zandonai A, Bonatto D, Cantarelli Machado D, Pêgas Henriques JA. Differentiation of human adipose-derived adult stem cells into neuronal tissue: does it work? Differentiation. 2009 Mar;77(3):221–8.

10. Marei HES, El-Gamal A, Althani A, Afifi N, Abd-Elmaksoud A, Farag A, et al. Cholinergic and dopaminergic neuronal differentiation of human adipose tissue derived mesenchymal stem cells. J Cell Physiol. 2018 Feb;233(2):936–45.

11. Rezaei F, Tiraihi T, Abdanipour A, Hassoun HK, Taheri T. Immunocytochemical analysis of valproic acid induced histone H3 and H4 acetylation during differentiation of rat adipose derived stem cells into neuron-like cells. Biotech Histochem. 2018 Oct 1;1–12.

12. Safford KM, Hicok KC, Safford SD, Halvorsen Y-DC, Wilkison WO, Gimble JM, et al. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun. 2002 Jun 7;294(2):371–9.

13. Zemel’ko VI, Kozhukharova IB, Alekseenko LL, Domnina AP, Reshetnikova GF, Puzanov MV, et al. [Neurogenic potential of human mesenchymal stem cells isolated from bone marrow, adipose tissue and endometrium: a comparative study]. Tsitologia. 2013;55(2):101–10.

14. Blecker D, Elashry MI, Heimann M, Wenisch S, Arnhold S. New Insights into the Neural Differentiation Potential of Canine Adipose Tissue-Derived Mesenchymal Stem Cells. Anat Histol Embryol. 2017 Jun;46(3):304-15.

15. Lim J-H, Boozer L, Mariani CL, Piedrahita JA, Olby NJ. Generation and characterization
of neurospheres from canine adipose tissue-derived stromal cells. Cell Reprogram. 2010 Aug;12(4):417-25.

16. Park S-S, Lee YJ, Lee SH, Lee D, Choi K, Kim W-H, et al. Functional recovery after spinal cord injury in dogs treated with a combination of Matrigel and neural-induced adipose-derived mesenchymal stem cells. Cytotherapy. 2012 May;14(5):584-97.

17. Sago K, Tamahara S, Tomihari M, Matsuki N, Asahara Y, Takei A, et al. In vitro differentiation of canine celiac adipose tissue-derived stromal cells into neuronal cells. J Vet Med Sci. 2008 Apr;70(4):353-7.

18. Roszek K, Makowska N, Czarnecka J, Porowińska D, Dąbrowski M, Danielewska J, et al. Canine Adipose-Derived Stem Cells: Purinergic Characterization and Neurogenic Potential for Therapeutic Applications. J Cell Biochem. 2017;118(1):58-65.

19. Chung C-S, Fujita N, Kawahara N, Yui S, Nam E, Nishimura R. A comparison of neurosphere differentiation potential of canine bone marrow-derived mesenchymal stem cells and adipose-derived mesenchymal stem cells. J Vet Med Sci. 2013 Jul 31;75(7):879-86.

20. Chung DJ, Wong A, Hayashi K, Yellowley CE. Effect of hypoxia on generation of neurospheres from adipose tissue-derived canine mesenchymal stromal cells. Vet J. 2014 Jan;199(1):123-30.

21. Lo Furno D, Mannino G, Giuffrida R, Gili E, Vancheri C, Tarico MS, et al. Neural differentiation of human adipose-derived mesenchymal stem cells induced by glial cell conditioned media. J Cell Physiol. 2018 Oct;233(10):7091-100.

22. Zhang H-T, Luo J, Sui L-S, Ma X, Yan Z-J, Lin J-H, et al. Effects of differentiated versus undifferentiated adipose tissue-derived stromal cell grafts on functional recovery after spinal cord contusion. Cell Mol Neurobiol. 2009 Dec;29(8):1283-92.

23. Ryu HH, Lim JH, Byeon YE, Park JR, Seo MS, Lee YW, et al. Functional recovery and
neural differentiation after transplantation of allogenic adipose-derived stem cells in a canine model of acute spinal cord injury. J Vet Sci. 2009 Dec;10(4):273–84.

24. Boulland J-L, Mastrangelopoulou M, Boquest AC, Jakobsen R, Noer A, Glover JC, et al. Epigenetic regulation of nestin expression during neurogenic differentiation of adipose tissue stem cells. Stem Cells Dev. 2013 Apr 1;22(7):1042–52.

25. Cheung Y-T, Lau WK-W, Yu M-S, Lai CS-W, Yeung S-C, So K-F, et al. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology. 2009 Jan;30(1):127–35.

26. Haskell GT, LaMantia A-S. Retinoic acid signaling identifies a distinct precursor population in the developing and adult forebrain. J Neurosci. 2005 Aug 17;25(33):7636–47.

27. Dhara SK, Stice SL. Neural differentiation of human embryonic stem cells. J Cell Biochem. 2008 Oct 15;105(3):633–40.

28. Tan B-T, Wang L, Li S, Long Z-Y, Wu Y-M, Liu Y. Retinoic acid induced the differentiation of neural stem cells from embryonic spinal cord into functional neurons in vitro. Int J Clin Exp Pathol. 2015 Jul 1;8(7):8129–35.

29. Zavan B, Vindigni V, Gardin C, D’Avella D, Della Puppa A, Abatangelo G, et al. Neural potential of adipose stem cells. Discov Med. 2010 Jul;10(50):37–43.

30. Voga M, Drnovsek N, Novak S, Majdic G. Silk fibroin induces chondrogenic differentiation of canine adipose-derived multipotent mesenchymal stromal cells/mesenchymal stem cells. J Tissue Eng. 2019 Dec;10:2041731419835056.

Figures
Figure 1

Canine ASCs grown in pre-differentiation medium STIM1 for 24 hours (A, B) and 48 hours (C, D). Scale bars are 200 µm (A, C) and 100 µm (B, D).
Canine ASCs grown in pre-differentiation medium STIM1 for 24 hours followed by growth in KEM differentiation medium for 1 (A) and 3 days (B). Scale bars are 200 µm.
Morphology of canine ASCs grown in pre-differentiation medium STIM1 for 24 hours followed by growth in NIMa (A, B, C), NIMb (D, E, F) or NIMc (G, H, I) differentiation media for 3, 6 or 9 days. Scale bars are 100 µm (A, C, H, I) and 200 µm in all other images.
Growth curves (A) and viability plots (B) for ASCs. (A) Growth curves for all six canine ASCs before and after the induction of differentiation. At time point 1 cells were seeded, time point 2 represents number of cells at the beginning of the treatment with pre-differentiation medium STIM1, which was applied for 24 hours, followed by the addition of NIMA differentiation medium and cellular numbers after 3 (time point 3) and 9 days of differentiation (time point 4). Experiments were performed in triplicates; the results were not statistically significant (p>0.05). (B) Average viability of male and female dogs derived ASCs before and after the induction of differentiation. Experiments were performed with all six ASCs cell lines in triplicates; the results were statistically significant between male and female derived cells for cell viability after 9 days of differentiation in STIM1 and NIMA media (p = 0.006).
Figure 5

Canine brain immunofluorescence stainings. NF-H (A), GFAP (B), TUBB3 (E), MAP2 (F), DAPI (C, G), merged A-C (D), merged E-G (H). Scale bars are 50 µm.
Figure 6

Expression of neural markers TUBB3 (a, c), NF-H (d, f) and GFAP (g, i) in canine ASCs grown in pre-differentiation medium STIM1 for 24 hours and differentiation medium NIMa for three days. Nuclei were counterstained with DAPI (b, e, h). Scale bars are 20 µm.
Figure 7

Expression of neural markers TUBB3 (A, C), NF-H (D, F) and GFAP (G, I) in canine ASCs grown in pre-differentiation medium STIM1 for 24 hours and differentiation medium NIMA for nine days. Nuclei were counterstained with DAPI (B, E, H). Scale bars are 20 µm.
Percentage of GFAP, MAP2 and NF-H positive cells following 3 or 9 days differentiation in NIMA medium. Average percentages are presented per female and male derived ASCs. Differences between sexes were not statistically significant.