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Research Article

Isolation and Characterization of Olfactory Stem Cells from Canine Olfactory Mucosa [1]

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Abstract

Olfactory stem cells have great potential in the treatment of neurodegenerative diseases and they are good candidates for cell therapy due to the easy accessibility of olfactory mucosa. The main objectives of this study were isolation, proliferation and characterization of olfactory mucosa stem cells that were further differentiated into olfactory neurospheres derived cells. When grown on poly-D-lysine with a serum-free culture medium supplemented with EGF (50 ng/ml) and FGF2 (50 ng/ml), olfactory stem cells gave rise to neurospheres. When grown in serum-containing culture medium newly plated spheres gave rise to olfactory neurosphere derived cells. Gene expression analysis revealed that, OCT4, SOX2, Nanog, Nestin, β-tubulin and NCAM were expressed in olfactory stem cells. While the mRNA expressions of Nanog, Nestin, Oct4, βIII-tubulin and NCAM were downregulated in neurospheres, the mRNA expression of SOX2 upregulated in neurospheres. According to the gene levels of neurospheres generated from olfactory stem cells, beta tubulin and NCAM gene expressions were upregulated, whereas OCT4, Nanog, Sox2 and Nestin mRNA expressions were downregulated in Olfactory neurospheres derived cells. Olfactory mucosa of canine is a suitable alternative source of stem cells and can be applied to cell therapy in neurodegenerative diseases.

Keywords: *Olfactory stem cell, Canine, Neurosphere, Pluripotent*

Köpek Olfaktorik Mukozasindan Olfaktorik Kök Hücrelerin Izolasyonu ve Karakterizasyonu

Özet

Olfaktorik kök hücreler nörodejeneratif hastalıkların tedavisinde büyük bir potansiyele sahiptir ve olfaktorik mukozaya kolay erişilebilirliği sayesinde hücre tedavisi için uygun bir adaydır. Bu çalışmanın amacı olfaktorik nörosfer kaynaklı hücrelere kadar farklılaştırılabilen olfaktorik kök hücrelerin izolasyonu, proliferasyonu ve karakterizasyonudur. Olfaktorik kök hücreler EGF (50 ng/ml) ve FGF (50 ng/ml) içeren serumsuz kültür vasatı içerisinde kültüre edildiğinde nörosferleri oluşturdular. Serum içeren kültür vasatında tekrar kültüre edildiklerinde olfaktorik nörosfer kaynaklı hücreleri şekillendirdiler. Gen ekspresyon analizleri OCT4, SOX2, Nanog, Nestin, β-tubulin ve NCAM genlerinin olfaktorik kök hücrelerinde eksprese olduğunu ortaya çıkardı. Nanog, Nestin, Oct4, β tubulin ve NCAM gen ekspresyonları nörosferlerde downregüle olurken, SOX2 geni upregüle oldu. Olfaktorik kök hücrelerin oluşturduğu nörosferlerin gen seviyeleri ile karşılaştırıldığında olfaktorik nörosfer kaynaklı hücrelerde β tubulin ve NCAM gen ekspresyonları upregüle olurken OCT4, Nanog, Sox2 and Nestin mRNA ekspresyonları downregüle oldu. Köpeğin olfaktorik mukozası uygun alternatif bir kök hücre kaynağıdır ve köpek nörodejeneratif hastalıklarında hücre tedavisi için uygulanabilir.

Anahtar sözcükler: *Olfaktorik kök hücre, Köpek, Nörosfer, Pluripotent*

INTRODUCTION

Neurogenesis takes place in three primary areas in the nervous system. These areas include: the subgranular zone, which supplies new granule cells to the dentate gyrus of the hippocampus; the subventricular zone, which supplies new interneurons to the olfactory bulb; and the olfactory neuroepithelia, which generates new excitatory sensory

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neurons that extend their axons to the olfactory bulb [1]. NSCs (Neural stem cells) that are derived directly from CNS (Central nervous system) tissue are considered to be safe and non-tumorigenic [2]. Furthermore, NSCs are excellent candidates for cellular transplantation therapy because they have been shown to replace the dead or dying neural tissues, elaborate trophic factors to rescue dysfunctional endogenous neurons, inhibit inflammation, and deliver therapeutic proteins in a widely disseminated manner [3-7]. However, harvesting such cells directly from the CNS is an invasive procedure with ethical considerations [8]. OM (Olfactory mucosa) of human and dog can easily be obtained from cribriform plate of ethmoidal bone with a non-invasive nasal biopsy [9-12]. Thus, stem cells derived from canine OM stand as a promising candidate for a source of autologous graft, due to their accessibility [10,12,13].

It is well known that new neurons are continuously generated from the stem cells in OM throughout life [14,15]. Neurogenesis within the OM is substantiated by niches of stem cells, located both in the OE (olfactory epithelium) and in the underlying olfactory lamina propria. Within the OE, two distinct populations of stem cells contribute to the neurogenic process, namely the HBCs (horizontal basal cells) and the GBCs (globose basal cells) [16]. A new stem cell population from mesenchymal stem cell family has been recently discovered in lamina propria of OM [17-19]. Lamina propria derived stem cells named as ecto-mesenchymal stem cells have attracted the interest of the researchers, having advantages of easily accessible location, a high proliferation rate, an ability to proliferate in long-term cultures and a tendency to differentiate into neural cells [20]. Therefore, OM has been considered to be an essential source for adult neural stem cells. Neural stem cells in OM are multipotent and can be grown into NSs (neurospheres), as well as further differentiate into neurons, astrocytes, and oligodendrocytes *in vitro* [21]. Thus the generation of NSs is often considered as a sufficient evidence for the existence of a stem cell.

The importance of the dog, being as a large animal model of human neurodegenerative diseases, has led to interest in the isolation and characterization of dog stem cells derived from various tissues such as adipose, bone marrow and amnion [22-24].

In this study, our aim was to isolate and characterize a stem cell population from canine OM as an alternative source of adult stem cells rather than bone marrow and adipose tissue for the treatment of neurodegenerative diseases in canine.

MATERIAL and METHODS

Cell Culture

OM was obtained from each dog according to previous description [25]. Primary cell culture was performed according to a previous report [10]. Briefly the mucosal biopsies were dissected under a stereomicroscope to remove cartilage fragments, blood vessels, connective tissues and non-olfactory mucosa. The remaining OM was rinsed three times with Hank balance salt solution (HBSS) with 1% PS (Peniciline and Streptomycine; Invitrogen; 15140122), and transferred (with minimal dissection) into a 35 mm petri dish containing HBSS with 1% PS. OM was cut into pieces of about 1 mm^3 with a scalpel blade for 1 min *(Fig. 1a,b)*, and by applying explants culture, the tissue was kept in culture flasks of 25 cm^2 at 37°C in High Glucose DMEM/F-12 medium (Biochrom, Cat. #FG-0445) supplemented with 10% fetal bovine serum (FBS, Biochrom Cat. #S0113), and 1% PS. After 7 days of incubation of the explants in culture medium, medium was begun to change every two days. These cells were confluents after 8 day of culture. At this time, the culture medium was aspirated and cells were washed with HBSS. Then, the cells were incubated with 1 ml of trypsin-EDTA solution (Gibco Cat #25200-056) for 5 min at 37°C. The separated cells were collected, centrifuged and re-plated at the rate of 1:2 for subculture. The medium was changed each two days up to the confluence of the cells in the flask.

Olfactory Neurosphere Formation and Growing

To form NSs, trypsinized cells were re-plated into culture T25 flask pretreated with poly-D-lysine and fed with DMEM/HAM F12 (Serum-free culture medium, Invitrogen #31331-028) supplemented with ITS-X 1%, (insulin, transferrin, selenium invitrogen #51500056); EGF (epidermal growth factor, 50ng/ml, R&D Systems Cat. #236-EG); FGF2(basic fibroblast growth factor, 50 ng/ml, Cat. #233-FB) and 1% PS as previously described [9]. In order to collect olfactory neurospheres lysates, the culture medium with the floating NSs was transferred to 15 ml tubes. Then, 2 ml of DMEM/HAM F12 was added to the flask, and with a micropipette, fluxes and refluxes were performed to release the NSs that were still adherents. These NSs in suspension were added to the same 15 ml tubes.

The tubes were centrifuged at 1.100 rpm for 3 min (Nuve NF 800R) and the supernatants were removed.

These were dissociated with trypsin, replated into T25 flasks and cultured in serum containing culture medium. These ONS (olfactory neurospheres derived) cells were then expanded by passage and these cells were stored in -80°C for Real Time PCR analysis.

Total RNA Isolation and mRNA Expression Levels of Genes by Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated by RNAeasy kit (QIAGEN) and cDNA was generated with a First Strand cDNA Synthesis kit (Thermo Scientific) at a total volume of 20 µl according to the manufacturer's instructions. Real-time quantitative

PCR was performed in a Strategene Mx3005P QPCR system (USA). Expression levels of target genes were normalized to the housekeeping gene β-actin (ΔCt). Gene expression values were then calculated based on the ΔΔCt method using the equation: RQ = 2–ΔΔCt. PCR amplification was performed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). The primer sequences used in PCR reactions and PCR conditions are described in *Table 1*. Each assay was performed in triplicate and repeated three times.

RESULTS

Each slice of OM was plated in T25 flask and fed with a serum- containing High Glucose DMEM/F-12 medium. After 5 to 7 days, OM derived cells started to grow out of the explant and proliferate *(Fig. 1c)*. The culture medium was totally renewed every 2 days. When the culture had reached confluency after 8 days, the cells were passaged and grown in T75 flasks to obtain large quantities of cells. In the new flasks culture, proliferation, expansion and cell clusters formations were also observed after passage. Primary cultures were mainly composed of elongated adherent cells *(Fig. 1d)*.

To assay the potential of OS (olfactory stem) cells for generatation of NSs, OS cells were plated onto poly-D-lysine coated T25 flask culture petri dishes filled with DMEM/ HAM F12 supplemented with ITS-X (1%), EGF and FGF2. OS cell generated NSs by the next day. As shown in *(Fig. 1e,f)*, the NSs had a spherical structure.

In order to assay their ability to differentiate into ONS cells, olfactory NSs were collected and re-plated in serum containing culture medium. The medium was totally renewed once every 2 days. ONScells rapidly proliferated as an adherent monolayer *(Fig. 1g,h)*.

mRNA Expression Levels of Genes in Olfactory Stem Cells, Neurosphere, Olfactory Neurospheres-Derived Cells

Real time PCR analysis showed that OS cells expressed pluripotent stem cell genes such as OCT4, Nanog, Sox2

Table 1.*Oligonucleotide primer sequences and PCR programs*
Tablo 1. *Oligonükleotid primer sekanslarıve PCR programları*

Transcripts	Primer Sequences	PCR Programs	Cycles
Nestin	F: 5′-5′-GAGAACCAGGAGCAAGTGAA-3′ R: 3′-TTTCCAGAGGCTTCAGTGTC-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	35
βIII-tubulin	F: 5′-GAGGGCGAGATGTACGAAGA-3′ R: 3′-CCTATGGTGGGAAAACAGGA-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	35
GFAP	F: 5′-CGAGTTACCAGGAGGCACTA-3′ R: 3′-TCCACGGTCTTTACCACAAT-5′	In.95˚C 5′/95˚C 10′-56˚C 30″-72˚C 1′	35
NCAM	F: 5′-AGGCAGAGCATAGTGAATGC-3′ R: 3′-AGGCTTCACAGGTCAGAGTG-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	35
NANOG	F: 5′-GAATAACCCGAATTGGAGCAG-3′ R: 3′-AGCGATTCCTCTTCACAGTTG-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	45
OCT4	F: 5′-GCAGTGACTATTCGCAACGA-3′ R: 3′-ATTTGAATGCATGGGAGAGC-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	35
SOX2	F: 5′-AGTACAACTCCATGACCAGC-3′ R: 3′-ATCATGTCCCGGAGGTC-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	35
GAPDH	F: 5′-TGACACCCACTCTTCCACCTTC-3′ R: 3′-CGGTTGCTGTAGCCAAATTCA-5′	In.94˚C 2′/94˚C 20″-55˚C 15″-72˚C 1′	35

Table 2. *Comparison of mRNA expression levels of genes*
Tablo 2. *Genlerin mRNA ekspresyon düzeylerinin karşılaştırılması*

Groups	mRNA Expression Levels of Genes (fold increase +/decrease -)						
	Oct4	Nanog	Sox2	Nestin	β Tubulin	NCAM	GFAP
1	1.0	1.0	1.0	1.0	1.0	1.0	-
2[a]	(-) 1.8	(-) 1.8	(+) 1.3	(-) 1.6	(-) 2.8	(-) 5.2	-
3	1.0	1.0	1.0	1.0	1.0	1.0	-
4[b]	(-) 2.1	(-) 1.1	(-) 3.4	(-) 1.1	(+) 1.4	(+) 3.3	-

a: *Compare to the group 1. Group 1: OS cell, Group 2: neurospheres generated from OS cells;* ***b:*** *Compare to the group 3. Group 3: neurospheres generated from OS cells, Group 4: olfactory neurospheres-derived cells*

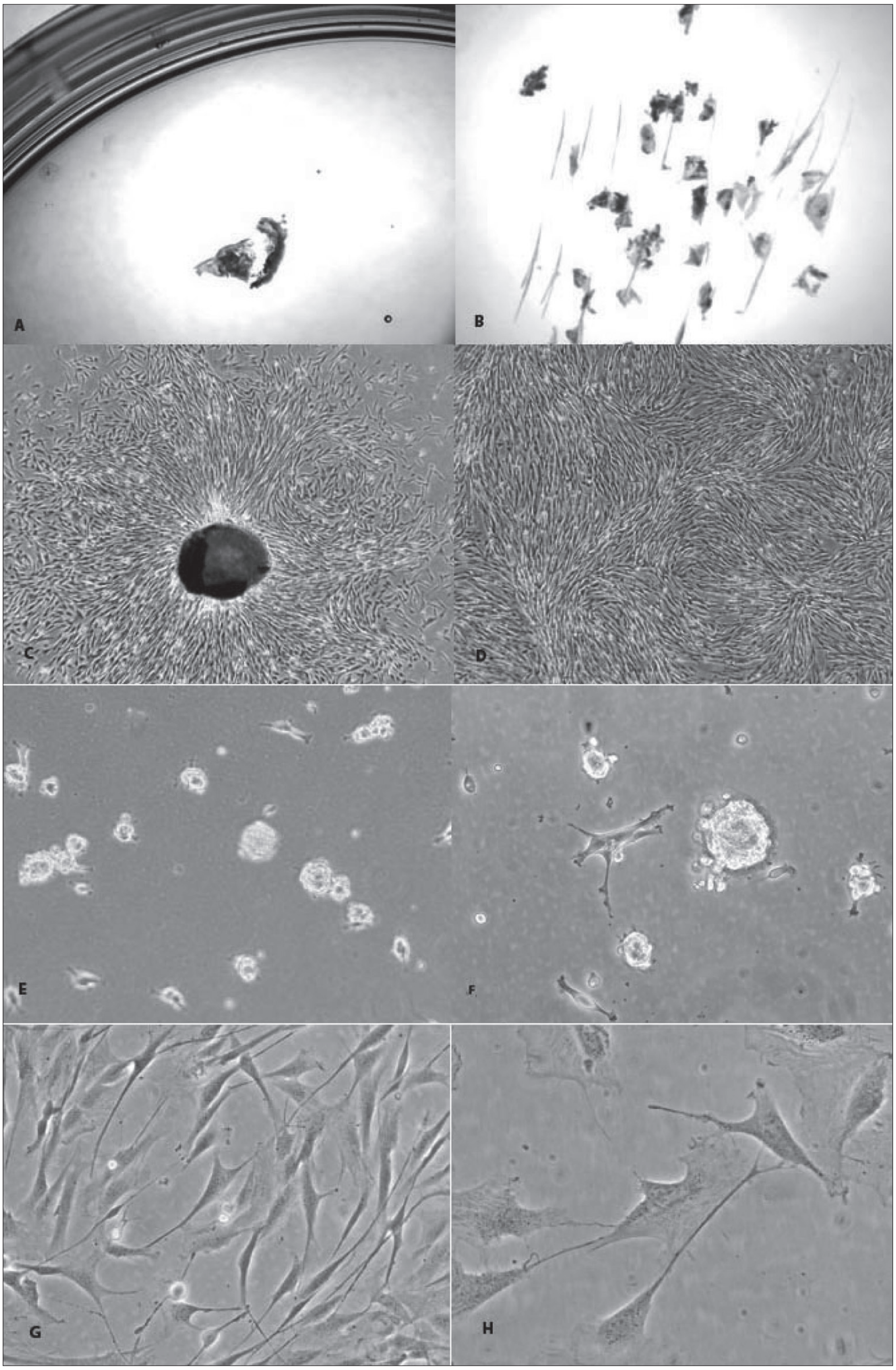

Fig 1. A. OM collection and B. OM tissue pieces of about 1 mm^3; C. cells adhesions close to the fragments; D. spindle shaped adherent cells; E and F. neurosphere formation; G and H. ONS cells

Şekil1. A. OM toplanması ve B. Yak-laşık 1 mm^3 büyüklükte OM doku parçaları; C. doku parçacıkların yakınında hücre tutunmaları; D. mekik şeklinde tutunan hücreler; E ve F. nörosfer şekillenmesi; G ve H. ONS hücreleri

genes, neural stem cell gene Nestin and neural specific genes NCAM, beta tubulin III, but the expression of astrocyte-specific gene that was GFAP was not present *(Table 2)*.

The expression of the neural stem cell marker Nestin was downregulated in the NSs derived from OS cells. Also, beta tubulin and NCAM expressions were determined to be downregulated in the NSs. GFAP was detected in neither NS nor ONS cells as in OS cells. In particular, the expression of pluripotent stem cell markers OCT4 and Nanog was downregulated in NSs when compared with the mRNA levels in OS cells. But the expression of another pluripotent stem cell marker Sox2 was found high in the NSs *(Table 2)*. According to the gene levels of NSs generated from OS cells, beta tubulin and NCAM gene expressions were upregulated, whereas OCT4, Nanog, Sox2 and Nestin mRNA expressions were downregulated in ONS cells *(Table 2)*.

DISCUSSION

OM generates 2 distinct types of NSs as follows: mesenchymal-like olfactory stem cells from lamina propria and epithelial-like OS cells from OE [9,20]. OE stem cells are similiar to olfactory propria stem cells with their identical primary cultures morphological appearance, expression of the stemness marker nestin or ability to form NSs which can subsequently proliferate as ONS cells or terminally differentiate into neuron-like cells [20]. Thus, we did not need to separate OE from lamina propria to isolate stem cells from each other. We collected canine OM according to a previous report [25] and to obtain OS cells, we chosed the non-enzymatic method in which the tissue slices left undisturbed for five days caused adherence of the explants to the plastic surface and subsequent emergence of the cell population [26]. Some researchers reaffirmed our approach [17,27] that nonenzymatic methods conserved the quality of the both OS cells and olfactory ensheating cells. Explantation causes minimal trauma to the tissue which is critical for cell quality. Within 5-7 days of explant culture, the spindle-shaped cells were observed migrating out of tissue explant onto the culture dish. Similar observations were observed by Alves et al.[10]. The migrated cells possessed typical mesenchymal morphology and continued to proliferate till 7 days. The cells were maintained in culture for a total of 15 days to attain confluence. When the culture reached confluence, the cells were passaged and transfered into the new culture flask. We observed OS cells in the form of elongated- spindle shaped morphology in the culture flask.

Various culture conditions generate NSs [19,28-32] that differ varying by species (human, rat, mouse), developmental stage (embryo, neonatal, adult), presence of serum, chemicals, and trophic factors. Here we used a serum-free culture method (supplemented with ITS, EGF and FGF2) to generate NSs from adult canine OS cells, because stem cells differentiated in the presence of growth factors and serum-free medium are safer for clinical use [24]. At the same time EGF has been demonstrated to be a mitogen for neural stem cells as has FGF2, and both factors in combination have been used to expand neural stem cells [16]. Also FGF2 causes growth in neural tube formation [33]. Thus, EGF and FGF2 together resulted in the most neurospheres forming [16]. Once the OS cell reach confluent in serum containing culture medium, they were plated on poly-D-lysine coated T25 flask that was filled with serum free culture medium supplemented with ITS, EGF and FGF2 at a 1:2 split ratio. The following day, OS cells had given rise to NSs under neurospheres forming conditions. Optimal cell plating density for NS formation is important. Optimal plating cell density should be 16.000 cell/cm^2 according to Girard et al.[9] but it should be 50.000 cell/cm^2 according to Carvalho et al.[20]. We got the best results for NS formation when we cultured the stem cells at a 1:2 split ratio. In this way, we successfully made OS cells turned into neurospheres in one day. In the present study, the expressions of the neural stem cell marker Nestin as well as the neural precursor markers βIII-tubulin and NCAM were detected in canine OS cells. In previous studies [23,24,34], these markers were also found in mesenchymal stem cells which were consistent with our findings. On the otherhand, we could not determine the GFAP mRNA expression in stem cells consisted with results of the study of Chung et al.[24]. Furthermore, ecto-mesenchymal stem cells (also named "mesenchymal-neural" precursors) have the capacity to differentiate into ectoderm and mesoderm cell types. Accordingly, ecto-mesenhcymal stem cells can also be expressing these neural markers in olfactory mucosa. At the same time, co-expression of neural markers such as Nestin, βIII-tubulin and NCAM except for GFAP shows high potential of canine OS cells to differentiate into multiple neural lineage in vitro. Nestin is expressed not only in nervous system organs such as olfactory mucosa but also in other organs and tissues. This may be the evidence that nestin-containing cells are pluripotent and may not be exclusively of neuroepithelial origin, such that nestin cannot be unambiguously interpreted as a marker of neural stem/progenitor cells [35]. Additionally, olfactory stem cells differ from bone marrow stem cells by over-expressing CD9 and under-expressing CD146 and CD200 [17]. CD9 belongs to the tetraspanin family and is considered as a pluripotency marker [36]. Afterwards Chaker N. Adra discovered and patented [37] that pluripotent stem cell populations could be obtained from olfactory mucosa and reported that some cells of the pluripotent stem cell population could differentiate into cells of one or more various lineages such as mesenchymal lineages or neuronal lineages or both. Thus, in this study, we also investigated the expression of pluripotent genes such as OCT4, Nanog and Sox2 because canine embryonic stem cells were demonstrated to express Oct4, Nanog, and Sox2 at high levels [38]. Sox2 governs ESC specification to neuroectoderm while Oct4 and Nanog promote their differentiation to mesendoderm, a common precursor of mesoderm and definitive endoderm [39], and Sox2 is a critical factor for directing the differentiation of pluripotent stem cells to neural progenitors and for maintaining the properties of neural progenitor stem cells [40]. In this study, we observed expressions of these pluripotent genes in OS cells and detected the down-regulation of Nanog, Oct4 and the upregulation of Sox2 in the NSs derived from OS cells. Furthermore, Nestin expression was downregulated in neurospheres and ONS cells consisted with the results of Carvalho et al.[41]. NSs contain a mixed population consisting of neural stem cells, proliferating neural progenitors and postmitotic neurons and glia [41]. Thus, in this study, the downrgulation of Oct4, Nanong and Nestin and the upregulation of Sox2 in NSs derived from OS cells indicated that there was a decrease in pluripotent characteristics of NSs and NSs highly expressing Sox2 gene have a high potential for differentiating towards functional neurons. Olfactory NSs were collected, dissociated and rapidly grown in the presence of serum as an adherent monolayer of ONS cells. These ONS cells had a flattened and undifferentiated appearance with a marker phenotype similar to the ectomesenchymal cells derived from primary cultures of OM [17,35]. That is, they expressed markers of mesenchymal stem cells (CD105 and CD73) as well as other stem and progenitor cell proteins (Oct4, Nestin, and beta tubulin) [35]. In this study, we demonstrated that ONS cell expressed a lower level of pluriptency genes and a higher level of neuronal genes compared with that of NS. Our findings highlight that pluripotent stem cells could be isolated from OM and they could be differentiated into NS and ONS cells, and therefore, these cells are suitable candidates for cell transplantation in neurodegenerative diseases of canine.

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