Dental, Oral and Maxillofacial Surgery

Periodontal ligament stem cell properties after long-term in vitro cultivation

Mihaylova Z.[1], P. Stanimirov[2], M. Miteva[3], N. Ishkitiev[4]

Изследване свойствата на стволови клетки от периодонтален лигамент след продължително култивиране in vitro

Михайлова З.[1], П. Станимиров[2], М. Митева[3], Н. Ишкитиев[4]

Summary

Purpose: The current topic in the field of regenerative medicine is the potential application of mesenchymal stem cells (MSCs) in the clinical practice. Some of the most commonly investigated MSCs are those of dental origin, due to the relatively easy access and non-invasive isolation protocol. However, there are reports in the literature revealing cell aging and senescence in MSC, after long-term in vitro cultivation. It is not clear whether these cells are going towards differentiation or degeneration, nor whether their regenerative potential is decreasing.

***Materials and methods:** We investigated cell senescence in human periodontal ligament (PDL) MSC after long term cultivation and managed to compare the stem cell properties of cells from early and late passages. For this purpose the cells were cultivated for a period of 1 week to 3 months.*

***Results:** When compared the stem cell properties in early and late passages PDL cells, no significant differences in their properties were found. All cell groups studied showed similar proliferation rate, markers expression and differentiation ability.*

***Conclusion:** We revealed no significant signs of aging and senescence in early and late passages PDL MSC.*

Key words*: periodontal ligament, mesenchymal stem cells, long-term cultivation, aging, senescence.*

***Резюме:** Денталната медицина става все по – сложна, което изисква прецизни двигателни умения и зрителна острота. През последните десетилетия тя търпи развитие както в клинично направление, но също така хистологичните аспекти играят важна роля в разработването на нови материали и оценка на лечебните процедури. В днешно време използването на увеличителни системи е популярна практика в денталната медицина. Това прави задължително опознаването на принципите на работа и физиката зад тези инструменти. Оперативният микроскоп е използван най-напред в офталмологията. Неговите ползи обхващат всички области на денталната медицина, включително оперативно зъболечение и ендодонтия, пародонтология, протетика, детска дентална медицина и имплантология. Изключвайки недостатъците като цена и маневреността на оборудването, увеличението се превръща във важен аспект на съвременната дентална медицина поради множеството предимства, които предлага. Този обзор отразява различните системи за увеличение,*

[1] Assistant Proffesor at Medical University-Sofia, Faculty of Dental Medicine, Department of Oral and maxillofacial surgery
[2] Associate Proffesor at Medical University-Sofia, Faculty of Dental Medicine, Department of Oral and maxillofacial surgery
[3] Student at Medical University-Sofia, Faculty of Dental Medicine
[4] Assistant Proffesor at Medical University-Sofia, Faculty of Medicine, Department of Med. Chemistry and biochemistry

[1] Асистент, МУ-София, ФДМ, Катедра Дентална, орална и лицево-челюстна хирургия
[2] Доцент, МУ-София, ФДМ, Катедра Дентална, орална и лицево-челюстна хирургия
[3] Студент, МУ-София, ФДМ, Катедра Дентална, орална и лицево-челюстна хирургия
[4] Асистент, МУ-София, МФ, Катедра Медицинска химия и биохимия

принципите им и прилагането им в различни области на денталната медицина.

Ключови думи: *увеличение, лупи, дентален микроскоп*

Introduction

Mesenchymal Stem Cells (MSC) are a multipotent cell population characterized by colony forming ability, expression of specific surface markers, and *in vitro* differentiation ability into various cell types - adipocytes, chondrocytes, osteoblasts [1]. They are expected to differentiate toward the cell type, identical to their tissue of origin. However, it has been reported that MSCs can also differentiate into cardiomyocytes, nerve cells, hepatocytes, etc. [2,3]. The *in vitro* cell culture environment significantly affects stem cell faith.

A few types of dental MSCs have been isolated and investigated [4]. These are stem cells from dental pulp of permanent teeth (DPSCs), stem cells from dental pulp of deciduous teeth (SHED), periodontal ligament stem cells (PDLSC), stem cells from apical papilla (SCAP), and dentate follicle stem cells (DFSC). Although, the long term cultivation ability of stem cells is known, the issue with the cell "immortality" and the length of life of MSC remains controversial and broadly discussed in the literature. Cellular aging is defined as progressive loss of cell resistance to stress, a gradual loss of cellular functions, cellular damage occurrence and eventually, resulting in cell death [5]. Normal somatic cells do not have unlimited potential for division and multiplication. They are characterized by the short replicative life, ending with cell arrest and/or death. Cultivated aging cells are unable of long-term DNA synthesis even in optimal conditions.

Cell aging is associated with a number of changes observed during the cell cycle phases. Some types of cells experience aging mainly during culturing under specific conditions [6]. This allows for two types of cell aging: spontaneously (due to normal cell cycle and shortening of telomeres) and premature (initiated by different environmental conditions). Wide range of factors can trigger premature aging of cells. These include a large number of mitogens (growth factors, cytokines), oncogenes, oxidative stress, cytostatic agents, toxic agents, DNA damage, and inadequate *in vitro* cultivation [7,8]. Most of these factors can also cause apoptosis in cell cultures, mostly depending on their concentration and time of cell exposure.

It is believed that MSC, like normal somatic cells, have a limited life span [9]. After a certain number of cell divisions, MSC enters the cell aging phase. Typical for this phase are morphological changes in cells such as increased size, irregular shape and limited proliferative capacity. These phenomena were first described in 1965 by Leonard Hayflick [9]. Recent reports reveal that the long-term *in vitro* reduces the MSC differentiation potential [10,11].

Nowadays, the molecular mechanisms underlying cellular aging are poorly understood. Two major hypotheses concerning stem cell aging are proposed: the first refers to a genetically determined duration of replicative life, and the second – to specific events within the cell and the influence of external factors [12].

On the other hand, unlimited cell proliferation and immortality of undifferentiated cells increases the risk of malignant transformation *in vitro* and *in vivo* [13]. Tumors are known to have cells with increased replicative potential [14]. In this sense, cell aging should be considered as a protective mechanism against the initiation of neoplasm development.

Many pathologies in the oral cavity are thought to be associated with cell aging and senescence, including periodontitis. Chronic generalized periodontitis is an inflammatory-degenerative disease that affects the tooth-supporting apparatus. All periodontal structures - periodontal ligament (PDL), alveolar bone, cementum and gingiva are involved. The incidence of chronic periodontitis increases with the age. Up

to 85-90% of the adult population suffers from periodontal diseases. Most of them have a mild to moderate form, and nearly 15% are affected by an advanced form of the disease [15].

Investigation of aging and senescence in periodontal MSCs would contribute to a better understanding of the diagnosis and treatment of one of the most common age-related diseases in dentistry – chronic generalized periodontitis. The purpose of the current article is to reveal the effect of long-term *in vitro* cultivation on cell proliferation, markers expression and differentiation ability in human PDL MSC. Early and late stem cell passages were analyzed and compared.

Materials and methods

1.Cell isolation and cultivation

Cells were obtained from PDL of intact surgically extracted third molars (n=5) of healthy patients (n=5) between 18 and 40 years old, after signing informed and written consent. PDL was scraped from the middle third of the roots with sterile scalpel blade and washed 3 times with Phosphate-buffered saline (PBS) (Lonza, Verviers, Belgium). Tissue explants were sliced into small pieces and enzymatically digested in a solution of 3mg/mL collagenase type I and 4mg/mL dispase (Sigma-Aldrich, St. Louis, MO, USA) for 15-30 min at 37°C and then transferred to 2 cm^2 culture dishes with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Eugene, OR, USA), supplemented with 1% antibiotic-antimycotic (Invitrogen) and 10% heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich). Cell cultures were incubated at 37°C in humidified atmosphere of 5% CO2 and 95% air for a long-term cultivation. The medium was replaced every 2nd or 3rd day thereafter until migrating cells colonies were established. Cell growth was monitored by phase contrast microscopy (Leica DMRE, Leica Microsystems GmbH, Germany). The explants were then moved to new culture dishes and the colonies of migrated pericoronal DFSC were detached with 0,05% trypsin/EDTA (Lonza, Basel, Switzerland) for 15 min. Then cells were transferred to 25cm^2 or 75cm^2 tissue culture flasks (TPP, Trasadingen, Switzerland) for further culturing. Cells were passaged when they reached 70-80% confluence. Experiments were conducted with cells between 1st and 10th to allow us to compare the properties of early and late cells passages and to find out the effects of aging due to long-term cultivation *in vitro*. Two to three months was the time needed for the cells to reach late passages. Each experiment was carried out at least 3 times.

2.Cell proliferation rate

When reaching sub confluence, PDL stem cells were detached from the bottom of cell culture flask and counted. Hemocytometer protocol was used to obtain a viable cell count from suspension. In the experiment, 10μL cell suspension was placed between the Hemocytometer and coverslip. One μL Trypan blue (Sigma Aldrich) was added to stain the apoptotic cells. Cells were counted under phase contrast microscopy. The Population Doubling level (PDL) was calculated with the following formula: 3.32(log (total viable cells at harvest/total viable cells at seed)).

3.Characterization of cell cultures

PDL cells were characterized for expression of surface and intracellular markers expression via immunocytochemistry. All experiments were carried out 3 times.

Antibodies used

Cells were incubated with the following non-conjugated antibodies: mouse anti-human alkaline phosphatase (ALP) (SigmaAldrich, USA), mouse anti-human vimentin, nestin, CD44H, DSPP (all Santa Cruz, Santa Cruz, CA, USA), mouse anti-human cytokeratine (CK)10 (AbCam, Cambridge, United Kingdom), rabbit anti-human CK19 (both AbCam). CFL488-conjugated goat anti-mouse and CFL555-conjugated goat anti-mouse, CFL488-conjugated goat

anti-rabbit and CFL555-conjugated mouse anti-rabbit (all SantaCruz) were used as secondary antibodies (1:1000). Cells were also incubated with FITS-conjugated mouse anti-human CD146 (Beckman Coulter International SA, Brea, CA, USA), at dilutions 1:1000.

Immunofluorescence

Five thousand cells/cm^2 were seeded in 96-well plates (TPP). After reaching sub-confluence cells were fixed with 4% paraformaldehyde for 30 min, washed 3 times with PBS and incubated with 1% BSA for another 30 min. For intracellular markers staining cells were permeabilized with 0,05% Tween20 (ICN Biomedicals Inc, Aurora, OH, USA) for 10 min and with 0,05% Triton X-100 (Calbiochem - Merck, Darmstadt, Germany) for 30 min, right after the fixation step. After incubation with antibodies and washing with PBS three times, cultures were incubated with DAPI (4,6-diamidino-2-phenylindole) (Invitrogen) for 15 min to stain the nuclei. Cell cultures were observed with two different instruments: the first one is a confocal scanning laser fluorescence microscope (Leica Microsystems GmbH) and the second one is IN Cell Analyzer 6000 imaging system-laser confocal slit system (GE Healthcare, Pittsburgh, PA, USA).

4. *In vitro* differentiation

In vitro cell differentiation of DF cell cultures was induced previously described methods [16] using osteogenic, chondrogenic and adipogenic cell culture media. Cells were incubated for a period of 3 weeks and the cell culture media were changed every 2nd or 3rd day. We used the following induction cell culture media: Osteogenic medium consists of 10mM β-glycerophosphate, 100nM dexamethasone and 50μM ascorbic acid; Chondrogenic medium consists of 100nM dexamethasone, 100μM ascorbic acid and 5ng/mL transforming growth factor (TGF) -β1; Adipogenic medium consists of 50μM dexamethasone, 500nM isobutyl methyl xanthine and 1μg/mL insulin. After 21 days of differentiation, the cells were fixed with 4% Formalin. Fixed cells were incubated with 2 g/mL Alizarin Red S (AppliChem GmbH, Darmstadt, Germany) with pH 4,2 to determine osteogenic differentiation, Alcian blue (Sigma Aldrich) for chondrogenic differentiation, Oil Red-O (Sigma Aldrich) for adipogenic differentiation.

Results

Cell count

After reaching 85-90% confluence, the cells were counted in order to reveal the cell proliferation rate in human PDL MSC culture. The population doubling time was calculated in order to precisely reveal the distribution of the cell number in the different passages. The cells were cultivated for a period of 2 to 3 months. This time was needed to obtain cells from 10th passage. After long term cultivation (from 1st to 10th passage) we found no statistically significant decrease in the cell count (Fig.1). Trypan blue staining did not reveal significant number of apoptotic cells. No more than 6 to 8 apoptotic cells were identified in each new passage.

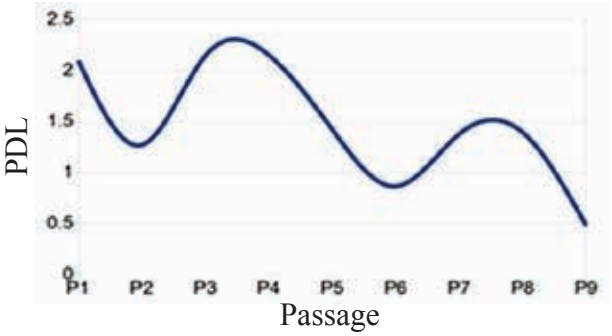

Fig.1 *Population doubling level of PDLMSCs; the following formula was applied: population doubling level=3.32(log(total viable cells harvest/total viable cells at seed))*

Markers expression

We compared the expression of stem cell and differentiation markers in periodontal MSC from early passages (1st to 3rd) and late passages (9th and 10th). When identifying the results from immunofluorescence staining, we found no dif-

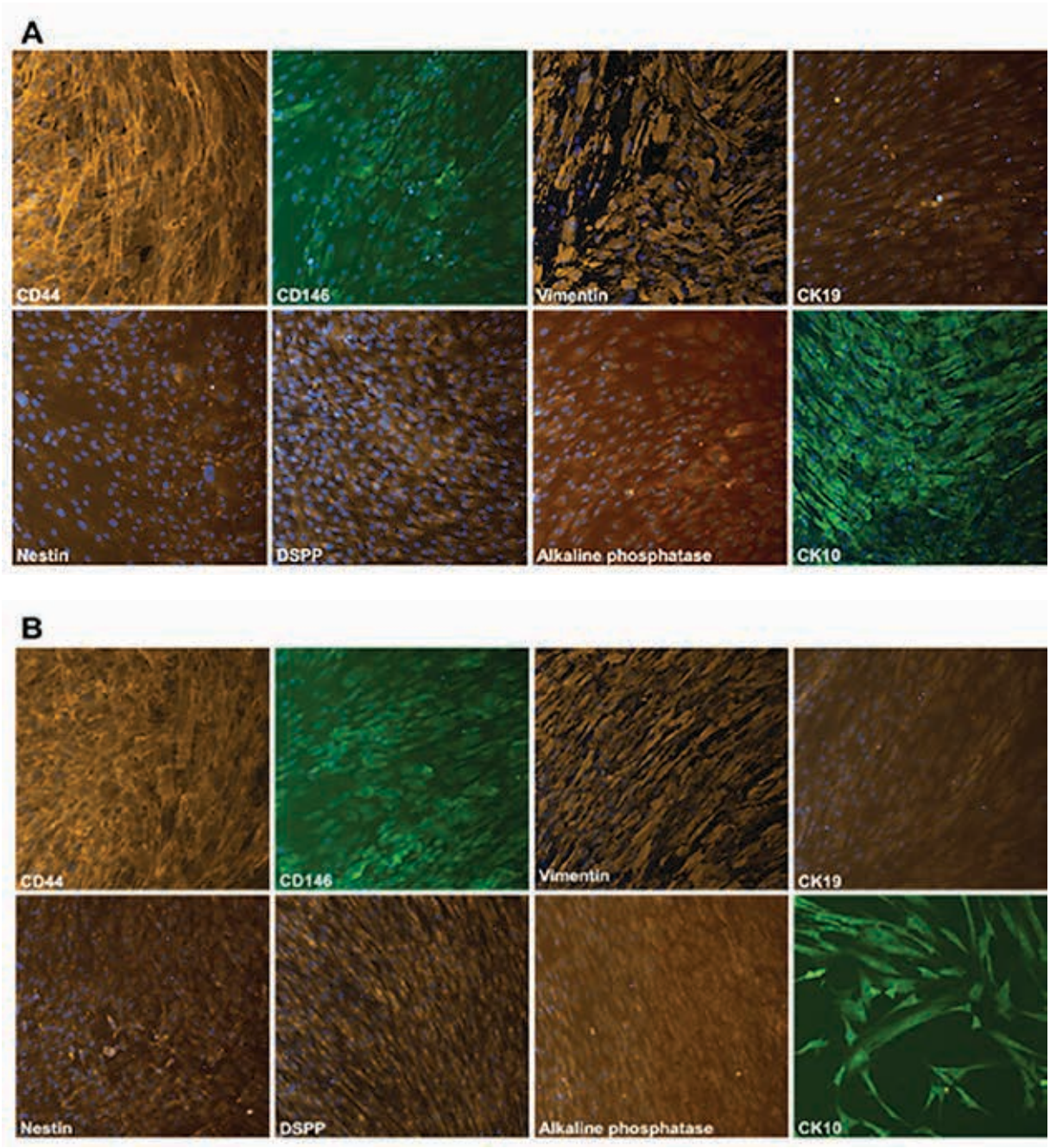

Fig.2 *Fluorescent marker expression characterisation of early passages (A) and late passages (B) of PDLSCs. (x20, Blue - DAPI).*

ference in the expression intensity between the groups of cells according to the culture time (Fig.2). No signs of aging or cell senescence were revealed.

In vitro cell differentiation

Late passages PDL MSC were cultivated *in vitro* for 3 weeks, in culture media supplemented by specific pre-defined protocols for stimulating adipogenic, chondrogenic and osteoblastic cell differentiation. We found strong Alizarin red and Alcyan blue staining, revealing the cell ability of osteogenic and chondrogenic differentiation, respectively. Oil red-O staining for lipid droplets was also found, but it was more limited.

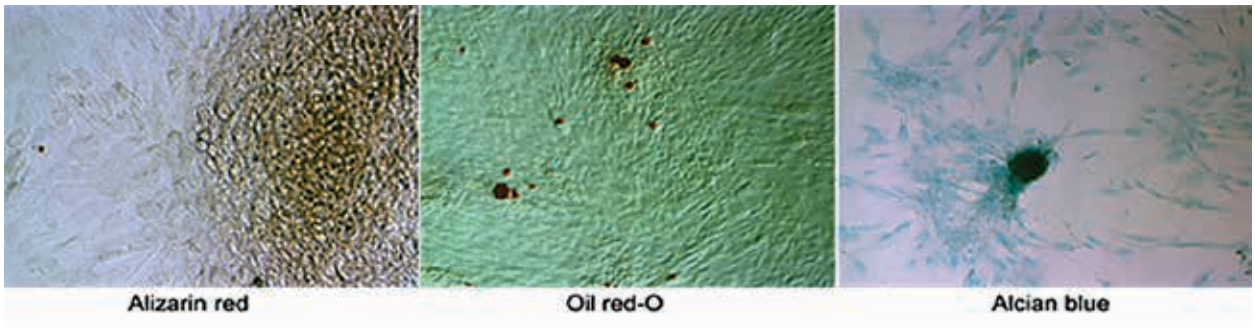

Fig.3 *Differentiation of normal PDLSCs cultures.(x5).*

Discussion

Aging is a physiological course of all living species, organs and cells. Multiple chronic diseases, characterized by impaired organ`s function related to senescence are observed. Impaired functions are associated with cell aging, followed by irreversible loss of self-renewal potential, differentiation and proliferation ability. However, little is known about the cell senescence in human MSC.

Aging is thought to be associated with advanced and severe clinical presentation of periodontitis in adults. However, the underlying mechanisms remain unknown. Age-dependent alterations in the immune system, as well as abnormal inflammatory status of the host are thought to be dependent on aging [17]. Reduction of the viable stem cell number in human PDL may significantly affect the outcomes of the cell regenerative therapy.

Wide range of studies report MSCs` ability of long-term cultivation and proliferation *in vitro* without any alteration in their stem cell phenotype. Although, studies revealing the PDL stem cell properties, in particular, commonly review the discrepancies between cell populations, delivered from young and elderly donors. According to Zhang et al. [18], the proliferative and migratory potential, and the differentiation capacity of PDL stem cells decrease with age. They concluded that these cells could be successfully delivered from donors of different ages, but the age-related changes of the MSC properties should be taken into account whenever they are intended for the purpose of research and especially if *in vivo* application is planned. To the best of our knowledge, there is lack of concise reports about the effects of long-term cultivation on various stem cell properties in human PDL MSC, referring to population doubling, multiple markers expression and multilineage differentiation.

In a study by Zheng et al. [19] is reported that PDL MSC obtained from aged donors exhibit decreased proliferation rate and differentiation potency when compared to those from young donors. Although, in a conclusion they stated that young stem cell-conditioned medium may enhance the stem cell properties of MSC obtained from aged donors. Further research is needed to clarify the stem cell properties.

In the current study we aimed to investigate the effects of long-term cultivation on various cell processes in human PDL MSC. Proliferation and apoptosis are the chief functions, maintaining the different cell functions. Therefore, we compared the cell proliferation rate (cell count, respectively) and apoptosis rate in early and late PDL cell passages. We found a decrease in the cell proliferation rate but without any statistical significance. No difference was detected in cell apoptosis, as we did not manage to find an increase in the number of apoptotic cells after long-term cultivation.

In another experiment we identified the expression of various markers in early and late stem cell passages. We investigated the expression of stem cell markers (CD44, CD146, nestin and vimentin), and differentiation markers associated with mineral tissues (DSPP, ALP) and epithelial phenotype (CK10, CK19). All markers were equally expressed in both cell groups. No

decreased or reduced markers expression was identified. The results confirmed the co-existence of undifferentiated stem cells together with cells of ectodermal and mesodermal origin, as the investigated cell culture is heterogeneous. However, the major number of cells had a mesenchymal stem cell phenotype.

In addition to the effect of the passage number on PDL MSC number, apoptosis and markers expression, we investigated its effects on cell functionality. Scarce adipogenic cell differentiation was found in elderly cell populations; although, the late passage cells demonstrate high osteo- and chondrogenic differentiation ability.

Our study shows no significant alteration in the stem cell properties of human PDL cell culture after long-term cultivation. However, further research is needed in order to deeply investigate the effects of various active substances on early and late cell passages, as well as the effects of cryopreservation when cells are prepared for *in vivo* application.

Conclusion

Long term cultivation related changes are usually taken into account whenever they are intended for use in research or in stem cell therapy. However, no statistically significant difference is found in the proliferative properties and differentiation ability of PDLSCs from early and late passages.

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References:

1. Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 2004; 36: 568-584.
2. Ishkitiev N, Yaegaki K, Imai T. High-purity hepatic lineage diferentiated from dental pulp stem cells in serum-free medium. J Endod 2012; 38: 475-480.
3. Ishkitiev N, Yaegaki K, Imai T, Tanaka T, Fushimi N, Mitev V, Ishikawa H. Novel Management of Acute or Secondary Biliary Liver Conditions Using Hepatically Diferentiated Human Dental Pulp Cells. Tissue Eng Part A 2014; 21: 586-593.
4. Akiyama K, Chen C, Gronthos S, Shi S. Lineage differentiation of mesenchymal stem cells from dental pulp, apical papilla, and periodontal ligament. Methods Mol Biol 2012; 887:111–121.
5. Mathon NF, Lloyd AC. Cell senescence and cancer. Nature Rev.Cancer, 2001;1:203–213.
6. Tang DG, Tokumoto YM, Apperly JA, et al. Lack of replicative senescence in cultured rat oligodendrocyte precursor cells. Science 2001;291:868–871.
7. Lundberg AS, Hahn WC, Gupta P, et al. Genes involved in senescence and immortalization. Curr Opin Cell Biol 2000; 12:705–709.
8. Roninson IB, Broude EV, Chang BD. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. Drug Resist Update 2001;4:303–313.
9. Hayfick L. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res 1965; 37:614–636.
10. Bonab MM, Alimoghaddam K, Talebian F, et al. Aging of mesenchymal stem cell in vitro. BMC Cell Biol 2006; 7: 14.
11. Noer A, Boquest AC, Collas P. Dynamics of adipogenic promoter DNAmethylation during clonal culture of human adipose stem cells to senescence. BMC Cell Biol 2007;8:18.
12. Hayfick L. Biological aging is no longer an unsolved problem. Ann N Y Acad Sci 2007;1100: 1–13.
13. Sager R. Senescence as a mode of tumor suppression. Environment Health Persp 1991; 93:59.
14. Kim NW, Piatyszek MA, Prowse KR. Specific association of human telomerase activity with immortal cells and cancer. Science 1994; 266(5193): 2011-2015.
15. Surget S, Khoury MP, Bourdon JC. Uncovering the role of p53 splice variants in human malignancy: a clinical perspective. Onco Targ Ther 2013;7:57–68.
16. Jeon BG, Kang EJ, Kumar BM, Maeng, et al. Comparative analysis of telomere length, telomerase and reverse transcriptase activity in human dental stem cells. Cell Transplant 2011; 20(11-12):1693-1705.
17. Hajishengallis G. Aging and its impact on innate immunity and inflammation:implications for periodontitis. J Oral Biosci 2014;56:30–7.
18. Zhang J, An Y, Gao LN, et al. The effect of aging on the pluripotential capacity and regenerative potential of human periodontal ligament stem cells. Biomaterials 2012;33(29):6974-6986.
19. Zheng W, Wang S, Ma D, et al. Loss of proliferation and diferentiation capacity of aged human periodontal ligament stem cells and rejuvenation by exposure to the young extrinsic environment. Tissue Engin Part A 2009;15(9):2363-2371.

Address of correspondence:
Zornitsa Mihaylova, Dept. OMFS, FDM, MU – Sofia 1431 Sv. G. Sofiyski 1; e-mail: zorry-mihaylova@gmail.com; GSM:0882492209