Original Article

Enzymatic Isolation, Amplification and Characterization of Dental Pulp Stem Cells

(enzymatic digestion / dental pulp stem cells / trypsin / isolation)

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Abstract. The dental pulp represents an easily accessible source of adult dental pulp stem cells (DPSCs). The preferred approach to DPSC isolation is enzymatic digestion. However, the duration of the enzymatic activity is crucial. The purpose of this study was to isolate the DPSC populations using this method, characterize their biological properties and proliferation capacity, and to determine their ability to differentiate into mature cells. Before enzymatic digestion using 0.05% trypsin, we used the homogenization method in order to obtain a fine homogenate from the solid pulp tissue. The stem cells were cultivated in modified cultivation medium for mesenchymal adult progenitor cells containing 2% foetal bovine serum, growth factors and insulin-transferrin-selenium supplement. We were successfully able to isolate 10 populations of DPSCs. The vitality of DPSCs did not drop below 90 %. However, the DPSCs showed a significant decrease in the relative telomere length number with increasing passaging ($P < 0.05$). Isolated DPSCs highly expressed the CD markers: CD29, CD44, CD90, CD13, CD73 and CD166. In contrast, CD markers CD31, CD106, CD34 and CD45 were negative or low positive. We confirmed the high osteogenic and chondrogenic potential of the isolated stem cells. Isolated DPSCs did not show signs of cell degeneration or spontaneous differentiation during the entire cultivation. In addition, we were able to shorten the enzyme activity duration, and we were the first to demonstrate trypsin as the enzyme used for the enzymatic digestion method with the viability over 90 % of isolated DPSCs using this method.

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Abbreviations: α-MEM – α modification of minimal essential medium, ASCs – adult stem cells, CD – cluster of differentiation, CXCR4 – chemokine receptor type 4, DAB – 3,3'-diaminobenzidine, DAPI – 4'-6-diamidino-2-phenylindole, DPSCs – dental pulp stem cells, ED – enzymatic digestion, ESCs – embryonic stem cells, FCS – foetal calf serum, FITC – fluorescein isothiocyanate, HBSS – Hank's balanced salt solution, hMSC – human mesenchymal stem cell, ITS – insulin-transferrin-selenium supplement, MSCs – mesenchymal stem cells, OG – outgrowth method, PBS – phosphate-buffered saline, PD – population doublings, PE – phycoerythrin, qPCR – quantitative polymerase chain reaction, SCs – stem cells, *STRO1* – a gene for protein marker of mesenchymal stem cells, TGF-β1 – transforming growth factor β1.

Introduction

Populations of stem cells (SCs) play a vital role in tissue and organ regeneration and maintenance because of their unique features, such as unlimited self-renewal, multilineage differentiation potential and proliferation capacity. Based on their origin, they can be classified into embryonic stem cells (ESCs) and adult (postnatal) stem cells (ASCs). Postnatal stem cells are more often investigated and analysed in research, because they lack the ethical concerns linked to their embryonic counterparts (Potdar and Jethmalani, 2015). Several populations of ASCs have also been isolated from various dental tissues. The dental pulp represents an easily accessible source of adult dental pulp stem cells (DPSCs). DPSCs were isolated in 2000 for the first time (Gronthos et al., 2000) and since then, they have been representing a promising future for the cell therapy. Another advantage of DPSCs, as the source of ASCs, is the display of characteristics of both ectoderm and mesoderm due to their ectomesenchymal embryonic origins. In addition, the dental pulp structurally resembles the primitive connective tissue within the tooth structure called "niche", which regulates stem cell behaviour and keeps it similar to embryonic stem cell behaviour (Morrison and Spradling, 2008).

Tooth extraction is a common surgical procedure performed in local anaesthesia and can be performed anytime throughout adult life. The extracted tooth would normally end up as biological waste. The pulp tissues

from human wisdom teeth or first premolars represent the most common source for DPSC harvesting. Supernumerary teeth, mesiodents, or third premolars are less commonly used (Ferrúra et al., 2017). The third molars are usually indicated for extraction as prevention of repeatable inflammation of the soft tissues surrounding the partially erupted clinical crown of the tooth. Extractions occur later, when patients are between 16 and 25 years old, and therefore usually involve fully developed teeth. On the other hand, premolars are extracted shortly after their eruption during orthodontic therapy at the patient's age of 12 years, hence they usually do not have fully developed roots.

After tooth collection, the dental pulp is isolated from the tooth structure and minced into small fragments. From this point, there are two common approaches to DPSC isolation. They can be isolated either by the enzymatic digestion (ED) method (Gronthos et al., 2000; Karamzadeh, 2012) or by the outgrowth (OG) method (Spath et al., 2010; Karamzadeh, 2012). In the first approach (ED), fragments of the dental pulp are immersed into a solution of enzymes in order to obtain a single-cell suspension. The most frequently applied solution contains collagenase type I and dispase in ratio 1 : 1. Other possible enzymes are trypsin and DNase (Ferrúra et al., 2017). Alternatively, the second most used method (OG) is based on the overgrowth from the minced pulp tissues in cultivation flasks. Although the OG procedure is less technically demanding, the ED approach is more commonly used for DPSC isolation. The DPSCs extracted using the ED approach provide higher proliferation capacity and differential potential (Jang et al., 2016). However, the specific enzyme used has a profound impact on the isolated stem cells, especially on the proliferation rate and differential potential (Dastgurdia et al., 2018). Even the duration of the enzymatic activity is crucial, because prolonged time can cause cell damage.

The purpose of this study was to isolate the DPSC populations using the ED method, cultivate them for a long time, investigate their proliferation capacity, analyse the phenotype, cell viability during the entire cultivation, relative telomere length in chromosomes of DPSCs in different passages, and determine their ability to differentiate into mature cells, chondroblast-like cells, osteoblast-like cells and adipocytes.

Material and Methods

The guidelines for this study were approved by the Ethical Committee of the University Hospital Hradec Králové ref. 201812 S07P. Patients or their legal representatives, in the cases of underage patients, had been adequately briefed before they signed informed consents voluntarily.

Tooth extraction

Tooth extractions occurred in local anaesthesia and under complete aseptic conditions at the Dental Clinic of the University Hospital Hradec Králové. The sources of pulp tissues for DPSC harvesting were permanent teeth in different stages of root development. After each extraction, the microbial plaque from the tooth structure was wiped out using sterile gauze and the tooth was decontaminated in 0.2% solution of chlorhexidine gluconate for 30 s. Extracted teeth were transported to the culture laboratory at 4 °C, fully immersed in transportation medium composed of 1 ml of Hank's balanced salt solution (HBSS) (Invitrogen, Waltham, MA), 9 ml water for injection (Bieffe Medital, Grosotto, Italy) and antibiotics and antifungal agent preventing any potential contamination, including 200 µl/10 ml streptomycin (Invitrogen), 200 µl/10 ml gentamicin (Invitrogen), 200 µl/10 ml penicillin (Invitrogen), and 50 µl/10 ml amphotericin (Sigma-Aldrich, St. Louis, MO).

Dental pulp isolation

The dental pulp was retrieved using two approaches in a laminar box under strict aseptic conditions. In the case of extracted teeth with not fully developed roots, we isolated the pulp tissues through the wide-open apical foramina using a sterile sharp probe and tweezers. The foramina had to be approximately 2 millimetre open to retrieve the pulp using this method. For the fully matured teeth, the tooth had to be split at a cement enamel junction using Luer's forceps to open a pulp chamber and isolate the pulp tissues from the crown and root canals separately using a sterile sharp probe and tweezers as well (Fig. 1A, B).

Enzymatic digestion

The collected pulp tissues were cut into small fragments using sterile scissors and inserted into a mini tissue grinder (Radnoti, Covina, CA) with an isotonic solution (phosphate-buffered saline, PBS) to obtain a very fine homogenate (Fig. 1C). The minced homogenous pieces were transferred into a tube and enzymatically digested using 0.05% trypsin (Gibco, Thermo Fisher Scientific, Foster City, CA) for 10 min at 37 °C. Following centrifugation (5 min, 600 *g*), the obtained cell pellet was resuspended and seeded in a culture flask (Sarstedt, Newton, NC). The isolated populations of DPSCs were cultivated in modified cultivation medium (Alpha-MEM, Gibco) for mesenchymal adult progenitor cells containing 2% foetal bovine serum (FBS, PAA Laboratories, Darthmouth, MA) and supplemented with 10 ng/ml epidermal growth factor (PeproTech, London, UK), 10 ng/ml platelet-derived growth factor (PeproTech) and 50 mM dexamethasone (Bieffe Medital), 0.2 mM L-ascorbic acid (Bieffe Medital) for protection against oxygen radicals, essential amino acid glutamine (Invitrogen) in the final concentration of 2%, and antibiotics – 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen), 20 µg/ml gentamicin (Invitrogen) and 0.4 µl/ml amphotericin (Sigma-Aldrich). According to our previous study (Suchanek, 2017), the medium was also enriched with nutrient utilization. The culture flasks were cultivated in incubators at 37 °C, 5% CO_2

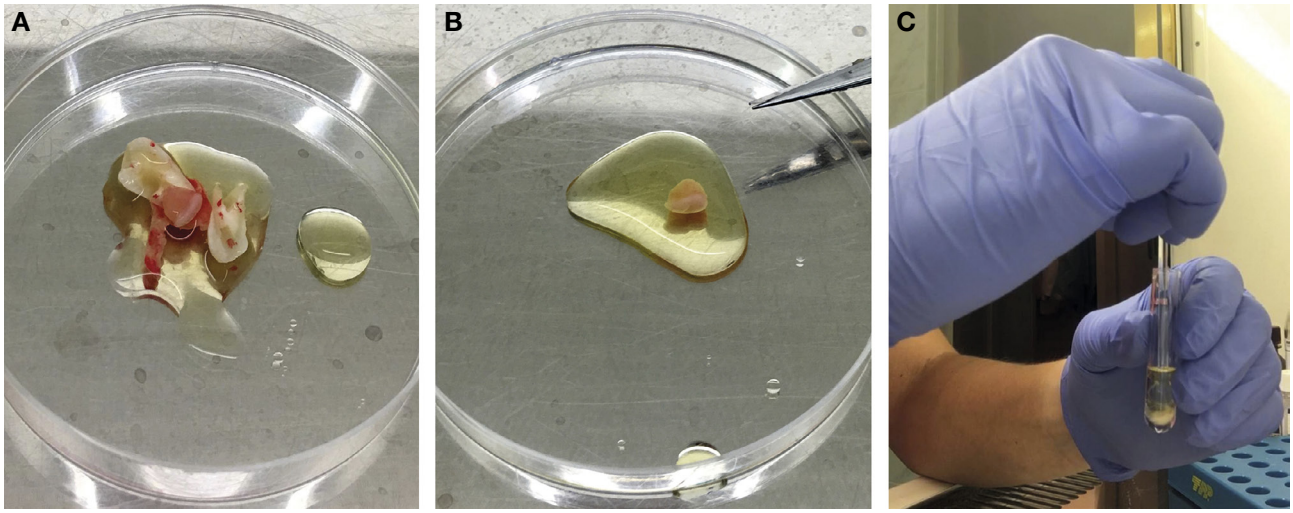

Fig. 1. Isolation of the pulp tissue and obtaining single-cell suspension
A. splitting the tooth at the cement enamel junction; **B**. dental pulp isolation from the tooth using a probe and tweezers; **C**. homogenization in a mini tissue grinder.

atmosphere. The culture medium was changed every three days until stem cells reached 70 % confluence, and then the cells were passaged and seeded in concentration 5,000 cells/cm^2 of cultivation flask. All DPSC populations were cultivated until they reached the 8th passage.

DPSC characteristics

During the entire cultivation, the cell count and cell diameter were measured using Z2-Counter (Beckman Coulter, Miami, FL). Cell viability was assessed based on the trypan dye exclusion method using the Vi-Cell analyser (Beckman Coulter) in the 2nd and 8th passages. Phenotype analysis was performed using flow cytometer Cell Lab Quanta (Beckman Coulter) in the 3rd and 7th passages. DPSCs were detached for the flow cytometry analysis and stained with immunofluorescence antibodies (FITC- and PE-labelled) against cluster of differentiation markers (CD). We examined the following markers: CD10 (CB-CALLA, eBioscience, Waltham, MA), CD13 (WM-15, eBioscience), CD18 (7E4, Beckman Coulter), CD29 (TS2/16, BioLegend, San Diego, CA), CD31 (MBC 78.2, Invitrogen), CD34 (581 (Class III), Invitrogen), CD44 (MEM 85, Invitrogen), CD45 (HI30, Invitrogen), CD49f (GoH3, Invitrogen), CD63 (CLBGran/12, Beckman Coulter), CD71 (T56/14, Invitrogen), CD73 (AD2, BD Biosciences Pharmingen, Erembodegen, Belgium), CD90 (F15-42-1-5, Beckman Coulter), CD105 (SN6, Invitrogen), CD106 (STA, BioLegend, USA), CD117 (2B8, Chemicon, Tokyo, Japan), CD133 (13A4, eBioscience), CD146 (TEA1/34, Beckman Coulter), CD166 (3A6, Beckman Coulter), CD271 (ME20.4, BioLegend), CD326 (G8.B, Invitrogen), HLA I (Tu149, Invitrogen). and HLA II (Tü36, Invitrogen), antiCXCR4 (12G5, Invitrogen), STRO1 (STRO-1, BioLegend). Classification criteria for CD marker expression were: < 10 % – no expression, 11–40 % – low expression, 41–70 % – moderate expression, > 71 % – high expression (Suchanek et al., 2010).

Quantitative PCR

Telomere length measurement was performed by the quantitative polymerase chain reaction (qPCR) assay according to the method described by Mokry et al. (2010) in the 2nd a 7th passages. Genomic DNA was extracted from the stem cells using a silica-gel-membrane-based DNeasy Tissue Kit (Qiagen, Hilden, Germany). ΔCt was determined using the equation: $\Delta Ct = Ct_{telomere} - Ct_{single\text{-}copy}$ gene. *36B4*, encoding acidic ribosomal phosphoprotein and often used as an internal standard for analysis, was used as the single-copy gene (a housekeeping gene). The relationship between relative telomere length in the 2nd a 7th passages was determined by Wilcoxon matched pair analysis using statistical software GraphPad Prism 5.01 (San Diego, CA). A value of $P < 0.05$ was considered as statistically significant.

DPSC differentiation

We used commercially available differentiation media to determine the ability of isolated DPCSs to differentiate into mature cell populations, namely osteoblast-like cells, chondroblast-like cells and adipocytes. The differentiation potential of DPSCs was examined in the 4th passage. DPSCs were seeded in the differentiation media in a monolayer after they reached 70–100 % confluence.

Chondrogenesis was induced using the Differentiation Basal Medium-Chondrogenic (Lonza, Basel, Switzerland) enriched with 50 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN) for 21 days. The chondrogenic medium was replaced twice a week. At the end of the differentiation protocol, chondrogenic pellets were fixed with 10% formalin and embedded in paraffin. To better understand the origin of the produced extracellular matrix after induced chondrogenesis, we used immunocytochemistry. Paraffin-embedded sections were deparaffinized, washed with distilled water, heated up in citric

buffer (Penta, Prague, Czech Republic) and washed with distilled water again. For permeabilization, which helps the antibodies to get into the fixed cells, we used 0.5% solution of Triton (250 ml Triton (Sigma-Aldrich) and 500 ml PBS) for 10 min. After thorough washing three times with PBS, cells were blocked in PBS containing goat serum (1 : 20, Jackson ImmunoResearch Labs, Cambridgeshire, UK) and then incubated in goat primary antibody (1 : 500, Sigma-Aldrich) directed against collagen type II at 4 °C for 60 min. After washing, cells were incubated with Cy3™-conjugated mouse IgM secondary antibody (1 : 300, Jackson ImmunoResearch Labs) at room temperature for 45 min. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 5 min. Samples were observed with a BX51 Olympus microscope (Olympus, Tokyo). Images were overlapped using Adobe® Photoshop CC 2019. As a comparison, the paraffin-embedded sections were also stained using the blue Masson's trichrome (collagen and procollagen stained blue).

Osteogenic differentiation was triggered using the Differentiation Basal Medium-Osteogenic (Lonza) for 21 days. Medium was changed every three days. After three weeks, differentiated stem cells with osteogenic nodules extracellularly were fixed with 10% formalin and embedded in paraffin. Paraffin blocks were cut in 7 μm thick slices and stained using the blue Masson's trichrome (collagen and procollagen stained blue) and the von Kossa stain (calcium particles stained black, DPSC nuclei stained red) to demonstrate the presence of osteogenic matrix and calcium deposits. We also did immunochemistry to quantify osteocalcin in the produced extracellular mass. Paraffin-embedded samples were deparaffinized and we used permeabilization to facilitate antibody penetration into cells and washed them in distilled water. After washing three times with PBS for 5 min, cells were blocked in PBS containing donkey serum (1 : 20, Jackson ImmunoResearch Labs) and then incubated in donkey primary antibody (1 : 50, Millipore, Kankakee, IL) directed against osteocalcin at 4 °C for 60 min. After washing, cells were incubated with mouse IgG secondary antibody (1 : 250, Jackson ImmunoResearch Labs) at room temperature for 45 min. After washing using PBS, samples were incubated in streptavidin-horseradish peroxidase (1 : 300, Dako, Glostrup, Germany). The samples were then stained using 3,3'-diaminobenzidine (DAB, Sigma-Aldrich). The oxidized DAB formed brown precipitates at the localization of the horseradish peroxidase. At the end of the protocol, the sections were stained with blue vitriol (Penta, Prague, Czech Republic) for 5 min and haematoxylin (Sigma-Aldrich) for 5 s. Finally, the samples were decalcified. We used a BX51 Olympus microscope with a digital camera for observation.

Differentiation in adipocytes was induced in a monolayer when DPSCs reached 100 % confluence either with hMSC Adipogenic Induction SingleQuots (Lonza) or with hMSC Adipogenic Maintenance SingleQuots (Lonza). Media were switched every three days for three weeks. After that, the cultures were fixed with 70% ethanol and stained with oil red (lipid particle vacuoles or droplets stained red).

Results

DPSC characteristics

For this study, we extracted ten adult teeth with different stages of root development from patients at the age of 13 to 18 years. We obtained five impacted or semi-impacted molars (from females at the age from 16 to 18 years), one impacted second molar (from a female at the age of 16 years), three first premolars (2 males and 1 female at the age of 13 to 14 years) and one supernumerary tooth (a mesiodent from a female at the age of 14 years). We were successfully able to isolate 10 populations of DPSCs. Although at the early stage of our cultivation we were able to yield just several tens of adherent cells, in four to five days we observed DPSCs forming small colonies. In a phase contrast microscope, most of the isolated stem cells had a spindle-like shape, elongated, flattened, with a couple of slim cytoplasmic processes (Fig. 2A). However, we also observed a more rounded shape in some adherent cells (Fig. 2B). The average cell diameter was 14.0 ± 0.8 μm. The viability of DPSCs did not drop below 90 % in any of the tested passages. The average viability was 92.2 ± 1.8 % in the 2nd passage and 93.00 ± 1.9 % in the 8th passage. The cell proliferation rate was characterized as cumulative population doublings and their doubling time, which is the period required for the DPSC population count to double (Figs. 3, 4). We observed a growth change during long-term cultivation. The highest and lowest cumulative population doublings reached were 51.6 and 40.8, respectively. At the beginning of cultivation, stem cells were more proliferatively active. In initial passages (from the 2nd to the 4th passage), the average doubling time was 39.4 ± 7.5 h. In general, the population time increased with increasing number of passages with some deviations. The average doubling time in higher passages (5th to 8th passage) was 62.9 ± 32.7 h. However, we observed that the populations Z03, Z04, Z05 reached extremely high doubling time in comparison to other isolated populations, even though their doubling times were similar at the beginning.

We terminated all cell populations in the 8th passage, and we did not witness any sign of spontaneous differentiation or cell degeneration. The phenotype analysis was performed in the 3rd and 7th passages. In total, we detected 25 clusters of differentiation markers using the flow cytometry method, but some of them were analysed just in three populations (CD49f, CD63, CD133, CD326, STRO1). Our isolated DPSCs showed high positivity for **CD13** (97.0 ± 1.3 % in 3rd passage and 95.00 ± 2.5 in 7th passage), **CD29** (90.2 ± 3.9 % in 3rd passage and 90.4 ± 3.9 % in 7th passage), **CD44** (95.2 ± 3.4 % in 3rd passage and 94.7 ± 3.2 % in 7th passage), **CD63** (81.7 ± 0.5 % in 3rd passage and 77.7 ± 2.3 % in

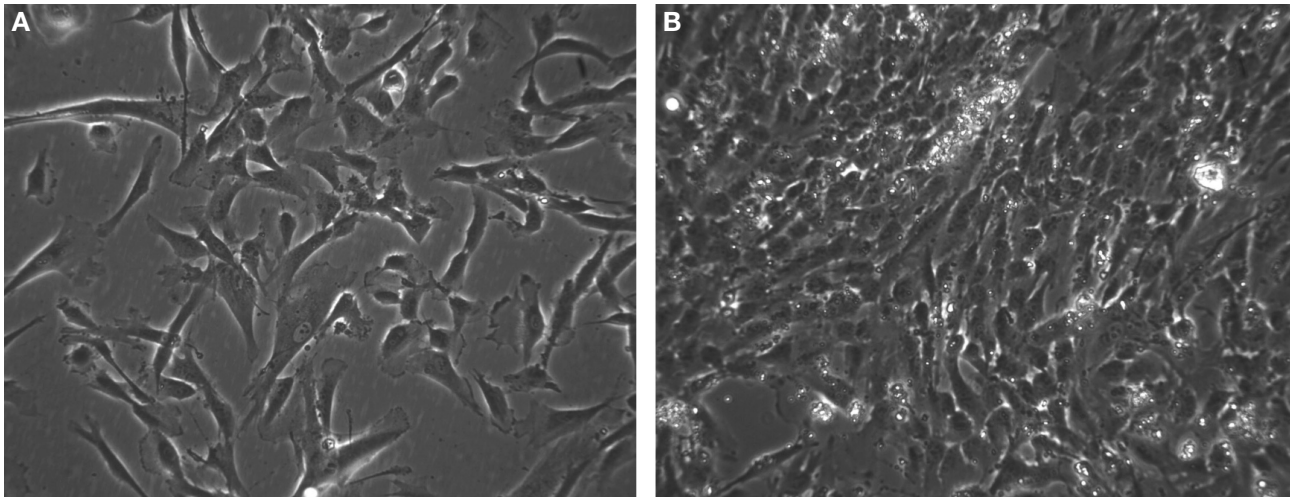

Fig. 2. DPSC primary culture. Phase contrast microscopy, direct magnification 200×
A. Spindle-shaped DPSCs with long cytoplasmic fibres; **B**. round-shaped DPCSs

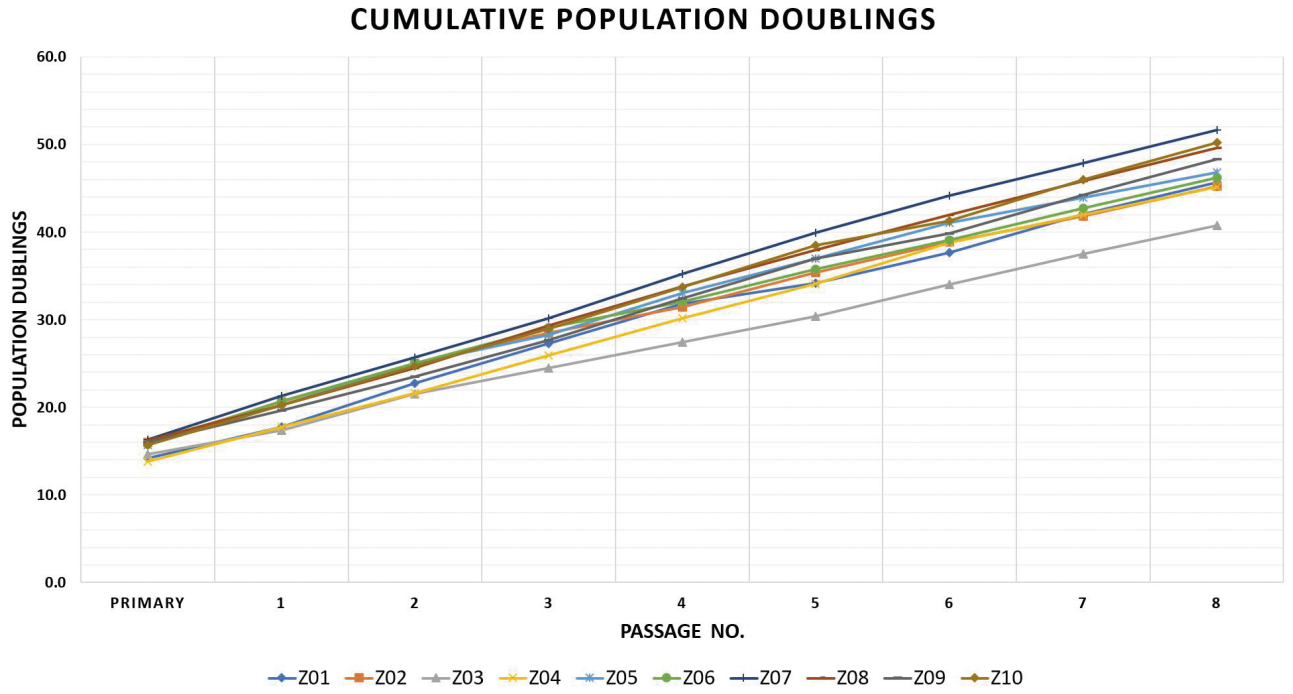

Fig. 3. Dental pulp stem cell population doublings (cumulative)

7th passage), **CD73** (92.8 ± 2.8 % in 3rd passage and 95.5 ± 2.6 % in 7th passage), **CD90** (97.5 ± 1.1 % in 3rd passage and 97.4 ± 1.6 % in 7th passage), **CD133** (81.5 ± 2.4 % in 3rd passage), **CD166** (89.5 ± 3.5 % in 3rd passage and 87.7 ± 8.8 % in 7th passage), and **CD326** (78.6 ± 1.7 % in 7rd passage). On the other hand, the following CD markers did not manifest themselves: **CD18** (0.6 ± 0.4 % in 3rd passage and 0.9 ± 1 % in 7th passage), **CD31** (1.4 ± 3.5 % in 3rd passage and 0.1 ± 0.2 % in 7th passage), **CD34** (1.3 ± 2.8 % in 3rd passage and 0.7 ± 0.6 % in 7th passage), **CD106** (1.0 ± 1.9 % in 3rd passage and 5.5 ± 9.0 % in 7th passage), **HLA II** (7.9 ± 7.7 % in 3rd passage and 8.1 ± 10.4 % in 7th passage), and **antiCXCR4** (4.5 ± 2.0 % in 3rd passage and 6.6 ± 3.3 % in 7th passage). **CD45**, a marker for haematopoietic stem cells, expressed itself in low positivity in both passages (16.4 ± 6.6 % in 3rd passage and 21.9 ± 7.0 % in 7th passage). The averaged expression of all CD markers is illustrated in Table 1. DPSCs showed a stable phenotype during the entire cultivation.

Relative telomere length

Table 2 shows the results of qPCR analysis of the isolated DPCSs in the tested passages. The relative telomere lengths of DPSCs collected in the 7nd passage were significantly lower than those in the 2nd passage.

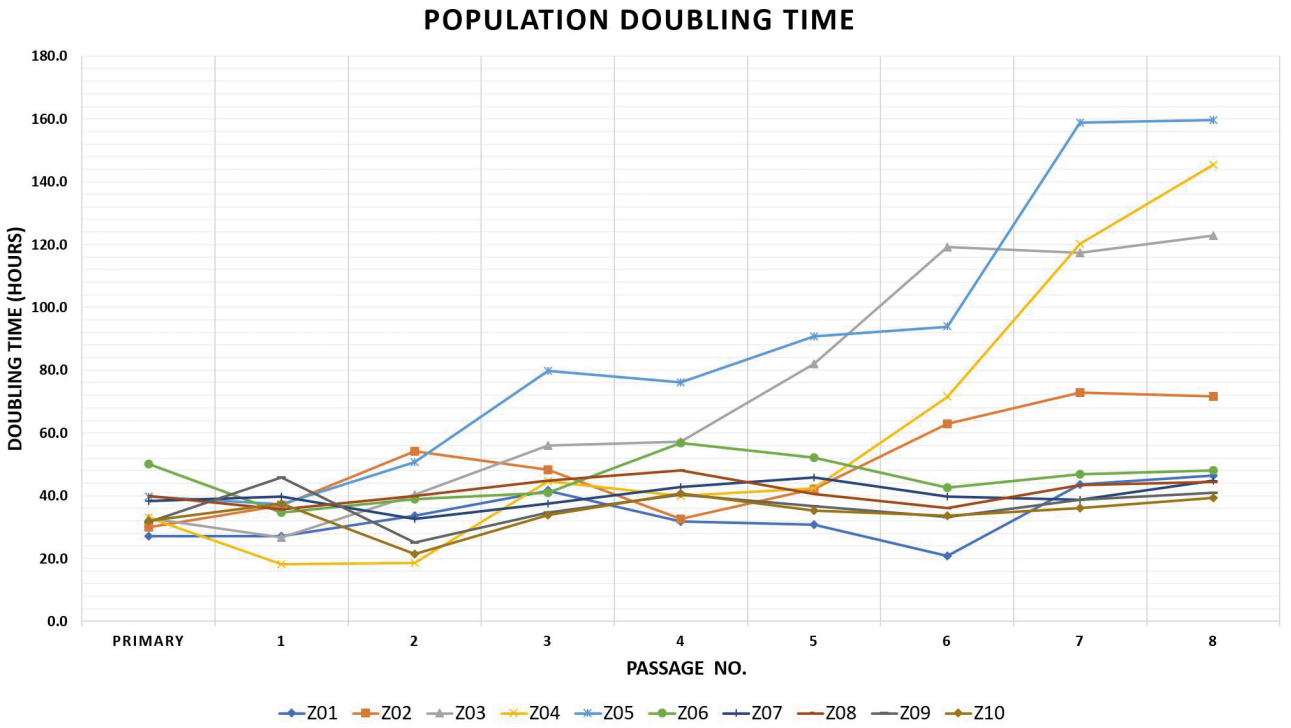

Fig. 4. Dental pulp stem cell doubling time trend

Table 1. Averaged expression in % of all CD markers in the 3rd and 7th passages

	3rd passage	7th passage		3rd passage	7th passage
CD10	23.8 ± 16.9	15.9 ± 19.7	**CD105**	55.3 ± 16.5	67.2 ± 14.1
CD 13	97.0 ± 1.3	95.0 ± 2.5	**CD106**	1.0 ± 1.9	5.5 ± 9.0
CD18	0.6 ± 0.4	0.90 ± 0.98	**CD117**	59.5 ± 11.3	47.4 ± 15.8
CD29	90.2 ± 3.9	90.5 ± 3.9	**CD133**	81.5 ± 2.4	
CD31	1.4 ± 3.5	0.1 ± 0.2	**CD146**	52.6 ± 21.9	59.4 ± 20.3
CD34	1.3 ± 2.8	0.7 ± 0.6	**CD166**	89.6 ± 3.5	87.7 ± 8.8
CD44	95.2 ± 3.4	94.7 ± 3.2	**CD 271**	14.5 ± 4.7	20.4 ± 5.5
CD45	16.4 ± 6.6	21.9 ± 7.0	**CD326**		78.6 ± 1.7
CD49f	46.6 ± 2.7	33.8 ± 2.7	**HLA I**	64.8 ± 19.9	48.5 ± 20.7
CD63	81.7 ± 0.5	77.7 ± 2.3	**HLA II**	7.9 ± 7.7	8.0 ± 10.4
CD71	15.2 ± 9.1	17.0 ± 5.0	**antiCXCR4**	4.5 ± 2.0	6.3 ± 3.3
CD73	92.8 ± 2.8	95.5 ± 2.6	**STRO1**	53.3 ± 2.8	32.3 ± 7.0
CD90	97.5 ± 1.1	97.5 ± 1.6			

Table 2. ΔCt of isolated populations of DPSCs in 2nd and 7th passages

Population	**Z1**		**Z2**		**Z3**		**Z4**		**Z5**		**Z6**		**Z7**		**Z8**		**Z9**		**Z10**	
Passage	2nd	7th	2nd	7th	2nd	7th	2nd	7th	2nd	7th	2nd	7th	2nd	7th	2nd	7th	2nd	7th	2nd	7th
ΔCt	–1.5	–1.7	–2.0	–1.7	–2.1	–1.0	–1.5	–1.0	–1.9	–1.3	–1.9	–1.5	–1.8	–2.1	–2.4	–1.8	–1.5	–1.4	–1.4	–1.1

The DPSCs showed a significant decrease in the relative telomere length number with increasing passaging ($P < 0.05$).

Multilineage differentiation potential

We tested the DPSC multipotency in the 4th passage. We exposed cells to osteogenic, chondrogenic and adipogenic inducing conditions. After three weeks, DPSCs

showed high potential to differentiate into osteoblast-like and chondroblast-like cells resulting in cell aggregation and production of osteogenic or chondrogenic pellets.

Using immunocytochemistry and fluorescent microscopy, we were able to demonstrate that the extracellular matrix contained collagen type II, the main component of cartilage. We also observed blue-positive areas in the stained samples using histological staining with blue Masson's trichrome (Fig. 5).

We were also able to observe nodules and three-dimensional structures formed by osteoblast-like cells in the extracellular matrix using a phase contrast microscope (Fig. 6A, B). Histological staining revealed the presence of collagen and procollagen fibres (Masson's blue trichrome staining, Fig. 7A) and calcium deposits (von Kossa staining, Fig. 7B, C) in the formed cell pellets. In addition, we confirmed the presence of osteocalcin using immunocytochemistry, a non-collagenous protein component of bone produced by osteoblasts. The positive immunoreaction appeared as brown precipitates in the produced extracellular matrix.

DPSCs cultivated in the adipogenic medium continued to proliferate and grow, but they did not show forming lipid vacuoles or accumulation of lipid droplets inside the cells. After oil red staining, the red-positive areas were very bright and invisible.

Discussion

Since 2000, when DPSCs were isolated for the first time, their popularity and interest in them have been increasing due to an easy access to them, high proliferation rate, and their ability to differentiate into multilineage mature cell populations.

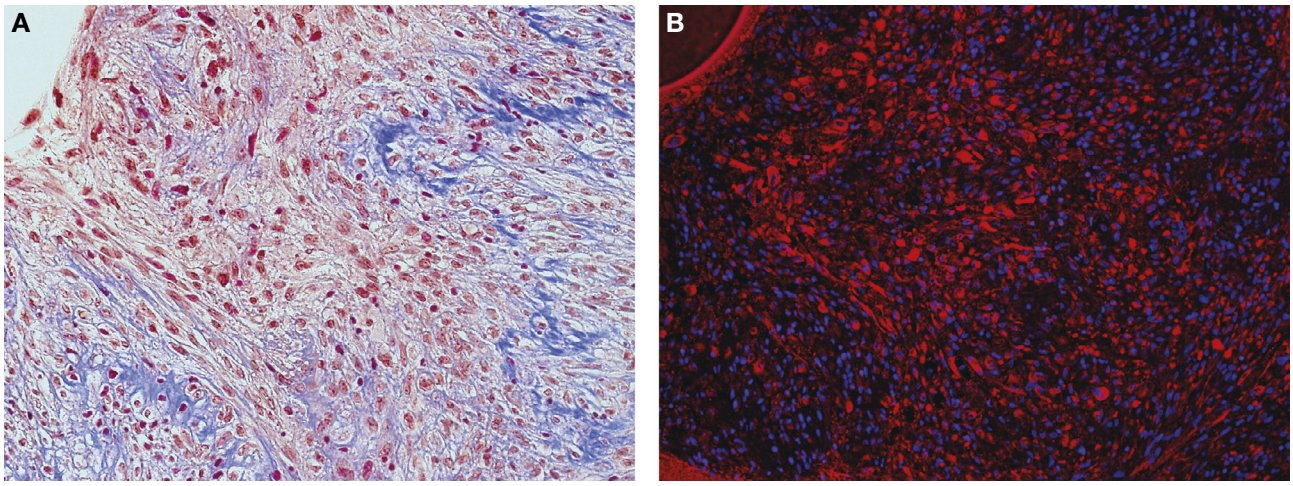

Fig. 5. Chondrogenic pellets of DPSCs cultivated in the chondrogenic medium for three weeks, direct magnification 200×
A. Masson's trichrome (collagen and procollagen stained blue); **B**. immunocytochemistry in fluorescent microscopy – collagen II immunoreactivity (red areas) and DAPI for cell nuclei (blue)

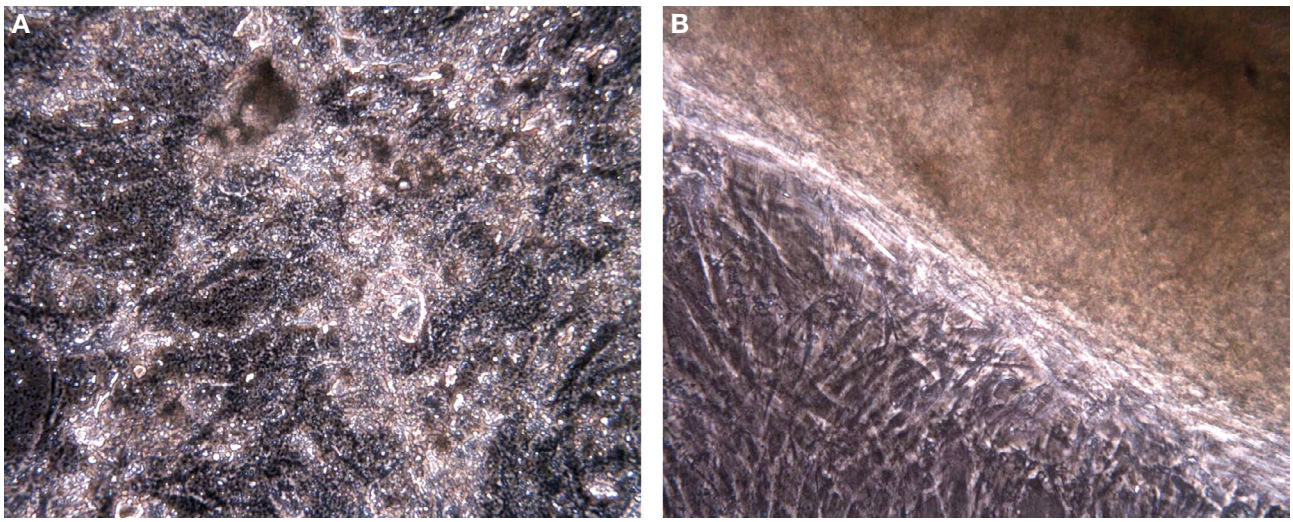

Fig. 6. Osteogenic pellets of DPSCs cultivated in the osteogenic medium for three weeks. Phase contrast microscopy, direct magnification 200×
A. Formed micro-mass in extracellular matrix; **B**. three-dimensional structure formed by osteoblast-like cells

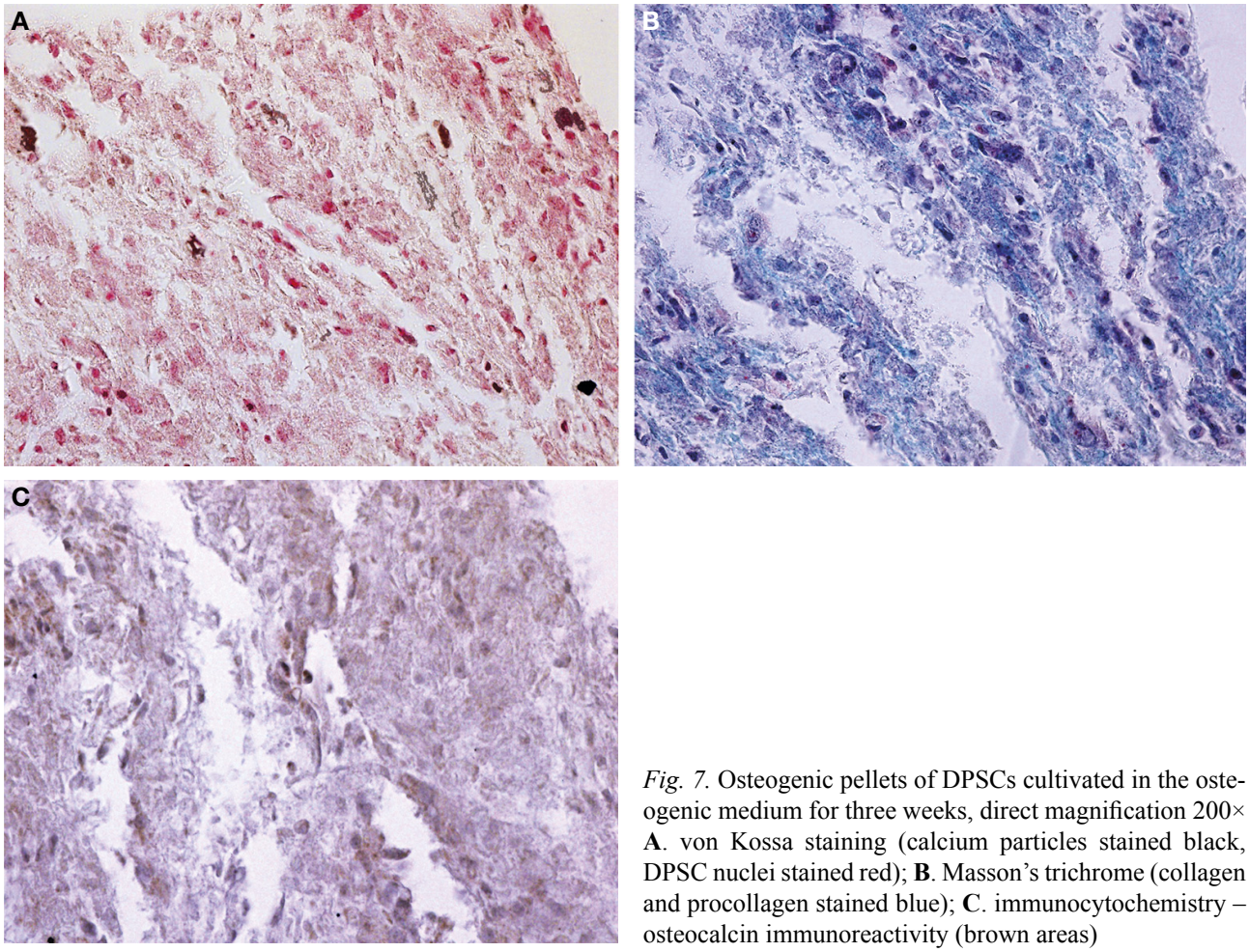

Fig. 7. Osteogenic pellets of DPSCs cultivated in the osteogenic medium for three weeks, direct magnification 200× **A**. von Kossa staining (calcium particles stained black, DPSC nuclei stained red); **B**. Masson's trichrome (collagen and procollagen stained blue); **C**. immunocytochemistry – osteocalcin immunoreactivity (brown areas)

Despite the fact that the overgrowth isolation method is less technically demanding, the enzymatic digestion is a more utilized approach for DPSC isolation worldwide. Ferrúra et al. (2017) reviewed more than 20 various combinations of enzymes used during the enzymatic digestion. The solution of collagenase type I, cleaving collagen I fibres (Fogarty and Griffin, 1973), and dispase, cleaving fibronectin and collagen IV (Kühn, 1995), is the most frequently used. The duration of the enzymatic activity of this combination, in ratio 1 : 1, is 30 to 60 min based on the size of the solid tissue that is being digested. In our study, we used 0.05% trypsin for 10 min as we harvested DPSCs enzymatically. In a tissue culture laboratory, trypsin is commonly used to resuspend cells adherent to the cell culture flask wall during the passaging of stem cells. To facilitate the enzyme action, we grinded the collected pulp tissues in a mini tissue grinder in order to obtain a very fine homogenate. The actual process of grinding is relatively simple and involves adding an extraction isotonic solution and the tissue to the mortar, and then slowly pressing the pestle with abrasive surface onto the sample with a twisting motion (Káš et al., 2005). The duration of the enzymatic activity is crucial in isolation of mesenchymal stem cells. The more prolonged is the activity time, the higher is the potential risk of damage to the harvested stem cells. In our study, we shortened the activity period of enzymatic digestion to 10 min using the homogenization technique and trypsin instead of a solution of collagenase type I and dispase in ratio 1 : 1. The isolated DPSCs showed no changes in viability, morphology or phenotype during the cultivation.

The isolated populations can be divided into two groups according to the achieved doubling time. We observed interesting deviations in the case of populations Z03, Z04, Z05. Their achieved population doubling times were extremely high in the end of their cultivation. This phenomenon cannot be explained by the type of the extracted tooth nor the age of the patients. All three populations were isolated from various teeth obtained from patients at different ages (Z03 from the second molar obtained from a female of the age of 16, Z04 from the first molar obtained from a female of the age of 14, and Z05 from the third molar obtained from a female of the age of 18). The populations were cultivated under the same culture conditions.

The isolated DPSCs highly expressed the CD markers for mesenchymal adult stem cells CD29, CD44, C90 and "stromal associated markers" CD13, CD73 and CD166. In contrast, CD markers for endothelial cells CD31 and CD106 were not expressed and haematopoietic markers CD34 and CD45 were negative or low

positive. The low positivity of CD45 can be explained by the cultivation of DPSCs in medium enriched with ITS, which keeps cells at a less differentiated level (Suchanek et al., 2013). We observed high proliferation capacity of the isolated cells.

In eukaryotes, during every DNA duplication the end of a chromosome is shortened, and thanks to that, the most distal part of each chromosome, telomere, is lost (Harley et al., 1990). The telomere is a non-coding repetitive nucleotide sequence at the end of each chromosome, which is responsible for chromosome protection against fusion, recombination or degeneration (Greider, 1996). However, uncompensated telomere loss leads to proliferation arrest and stops the cell duplication (Wong and Collins, 2003). This is assumed to be the reason for cell aging. Unlike somatic cells, stem cells possess a specific mechanism to compensate for this chromosome replication problem. They express the telomerase, a ribonucleoprotein enzyme that is responsible for the telomerase length maintenance. However, in our study, we observed telomere shortening with the increasing number of passages of the harvested DPSCs. This can be explained by the fact that such fast and remarkable *in vitro* cell amplification during the long-term cultivation may be associated with high stress conditions for the isolated stem cells and led to a decline in their replicative capacity. However, the high count of the obtained stem cells can be beneficial in the reparative or regenerative therapy.

We were able to trigger osteogenesis and chondrogenesis in the isolated DPSCs. We observed high production of osteogenic and chondrogenic cell pellets. As such, we confirmed the high osteogenic and chondrogenic potential of the isolated stem cells. Unfortunately, we were not successful in the initiation of adipogenesis. Not surprisingly, the adipogenic induction is more difficult for DPSCs, unlike for the mesenchymal stem cells harvested from the bone marrow. During aging, the bone marrow is physiologically replaced by lipid tissues. These observations correspond with the findings in Gronthos's study (Gronthos et al., 2002), where the authors concluded that DPSCs differentiated in adipocytes unwillingly.

Conclusion

We were successfully able to isolate 10 populations of DPSCs with no change in the cell morphology, phenotype, or with any signs of cell degeneration or spontaneous differentiation during the entire cultivation. In addition, we were able to shorten the period of enzyme activity during the DPSC isolation, and therefore reduced the potential risk of damage to the isolated cells. DPSCs showed high viability above 92 % on average and high proliferation capacity. At the same time, we observed a significant decrease in the relative telomere length with increasing passaging. We successfully initiated osteogenic and chondrogenic differentiation, but we were not able to trigger adipogenesis in the isolated DPSCs.

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