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# DENTAL PULP STEM CELLS AND THEIR CHARACTERIZATION

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**Aims:** Our aims were to isolate dental pulp stem cells, to cultivate them in various media and to investigate their basic biological properties and phenotype.

**Methods:** 16 lines of dental pulp stem cells (DPSCs) were isolated from an impacted third molar. After enzymatic dissociation of dental pulp, DPSCs were cultivated in modified cultivation media for mesenchymal adult progenitor cells containing 2 % or 10 % fetal calf serum (FCS), or in modified 2 % FCS cultivation media supplemented with ITS. Cell viability and other biological properties were examined periodically using a Vi-Cell analyzer and Z2-Counter. DNA analysis and phenotyping were done using flow cytometry.

**Results:** We were able to cultivate DPSCs in all tested cultivation media over 40 population doublings. Our results showed that DPSCs cultivated in medium supplemented with ITS had shorter average population doubling time (24.5, 15.55–35.12 hours) than DPSCs cultivated in 2 % FCS (55.43, 21.57–187.14 hours) or 10 % FCS (42.56, 11.86 – 101.3 hours). Cell diameter was not affected and varied from 15 to 16 μm. DPSCs viability in the 9$^{th}$ passage was over 90 %. Our phenotypical analysis was highly positivity for CD29, CD44, CD90 and HLA I, and negative for CD34, CD45, CD71, HLA II. DPSC lines cultivated in all media showed no signs of degeneration or spontaneous differentiation during the expansion process.

**Conclusions:** We showed that ITS supplement in the cultivation media greatly increased the proliferative activity of DPSCs. Other DPSC biological properties and phenotype were not affected.

## INTRODUCTION

The complex structural composition of teeth ensures both hardness and durability. However, these structures are vulnerable to trauma and bacterial infections. As ameloblasts are lost during eruption and odontoblasts can create new dentine only on a dentine-pulp border, a damaged tooth cannot self-repair. However teeth show a degree of reparative processes such as tertiary dentine formation. Once the odontoblast layer is damaged, odontoblast-like cells are recruited from somewhere within the pulp. Loss of the tooth, jawbone or both, due to periodontal disease, dental caries, trauma or some genetic disorders, affects not only basic mouth functions but aesthetic appearance and quality of life.

Current dentistry resolves these problems using autologous tissue grafts or metallic implants. These treatments have some limitations such as an adjoining tooth damage, bone resorption etc. The stem cell bioengineered tooth is a promising way of single tooth restoration. Some studies have reported that after dental pulp necrosis, dental pulp complex stem cells (DPSCs) can be used for the creation of dental pulp which after implantation into the shaped root canals has affinity for the dentine[1]. Moreover DPSCs can differentiate into dentine-pulp complex if they are induced by tooth germ cell conditioned medium[2].

Stem cells (SCs) are generally defined as cells which are able to selfrenew and to differentiate into various specialized tissues (e.g. fat, bone and cartilage, neural cells)[3,4]. Moreover, during in vitro cultivation, SCs are able to proliferate over the so-called Hayflick's limit[5]. Their main functions are tissue development, homeostasis and in the case of tissue damage, reparation. Multipotent mesenchymal stromal cells (MSCs) were found to be rare cells living in various mesenchymal tissues, for example in the bone marrow stroma (2 to 5 cells per million of nucleated cells)[6], liver or skeletal muscles[7]. More primitive MSCs were discovered later. These immunomagnetically separated cells were named mesodermal progenitor cells (MPCs)(ref.[8]) or multipotent adult progenitor cells (MAPCs)(ref.[9]). MSCs are a prospective source of cells for regenerative medicine. MSCs were also found within the dental pulp (DP). Dental pulp is a well-defined compartment of soft tissue which retains the primitive structure of gelatinous connective tissue. DPSCs were firstly identified by Gronthos et al.[10] in the year 2000. In later experiments these authors showed the ability of DPSCs to differentiate into various tissues (e.g. bone, cartilage) and their ability to self-renew. Miura et al. isolated stem cells from the dental pulp of exfoliated tooth[11]. DP could be an easily accessible source of histocompatible SCs.

The aims of this study were to compare phenotype and basic biological properties of DPSCs cultivated in different cultivation media. Using this approach we tried to find the best composition of cultivation media for DPSCs. For potential clinical uses of DPSCs (transplantation, regeneration) it is necessary to omit fetal calf serum from the media. For this reason, we decreased fetal calf serum concentration and used insulin, transferin, sodium selenite supplement.

## MATERIALS AND METHODS

We isolated 16 DPSCs lines from impacted third molars obtained from healthy donors. The average age of donors was 19 years (12–23, 12 females and 4 males). These or their legal representative gave written informed consent according to the guidelines of the Ethics Committee of the Medical Faculty in Hradec Králové.

Teeth were indicated for extraction because of orthodontic treatment or they cause severe healthy problems to the patients. Third molars were extracted under sterile conditions and transported to a tissue culture laboratory in 4° C cold Hank's balanced salt solution (Gibco, UK). If the tooth had fully developed roots we separated them from the crown using Luer's forceps. After separation of the roots or if the roots were not developed, we used an extirpation needle or sharp excavator (Henry Schein, UK) to isolate DP. Both dental pulp and the tooth were treated by enzymes – collagenase (Sevapharma, Czech Republic) and dispase (Gibco, UK) for 70 minutes. Following centrifugation (600 g, 5 min.) we obtained a cell pellet.

DPSCs were cultivated in 5 % $CO_2$ atmosphere under 37 °C in three different media. The first was composed of alfa-MEM (Gibco, UK), 2 % FCS (PAA, USA), 10 ng/ml EGF (PeproTech, USA), 10ng/ml PDGF (PeproTech, USA), L-ascorbic acid (Sigma, USA), 2 % glutamine (Gibco, UK), penicilin/streptomycin (Gibco, UK), gentamycin (Gibco, UK) and dexamethasone (Sigma, USA). The second was composed of alfa-MEM, 2 % FCS, 10ng/ml EGF, 10 ng/ml PDGF, L-ascorbic acid, glutamine, penicilin/streptomycin, gentamycin, dexamethasone and supplemented with 10 µl/ml ITS (Sigma, USA). The third (the most cited medium in the literature)[2,12] was composed of alfa-MEM, 10 % FCS, 1 % L-ascorbic acid, glutamine, penicilin/streptomycin and gentamycin. DPSCs were initially cultivated for 3-5 days in culture flasks with Cell$^+$ surface® (Sarstedt, USA). After this, small colonies were found and using trypsin-EDTA (Gibco, UK) we dissociated them and reseeded into standard tissue culture treated flasks (TPP, Switzerland or NUNC, Denmark). Each of the following passaging was done after reaching 70 % confluence.

Cell viability and number of population doublings were examined using Vi-Cell analyzer and Z2-Counter (both from Beckman Coulter, USA). DNA analysis (DPSCs passage No. 5 and 10) was done using propidium iodide staining and flow cytometry Cell Lab Quanta (Beckman Coulter, USA). Data were analyzed using Multi Cycle software (Phoenix Flow Systems, USA). For phenotypic analysis, cells (DPSCs passage No. 5) were detached and stained sequentially with immunofluorescent primary antibodies. The percentage of positive cells was determined as percentage of cells with higher fluorescence intensity than the upper 0.5 % isotype immunoglobulin control. Classification criteria: <10 % no expression, 11-40 % low expression, 41-70 % moderate expression and >71 % high expression. MPCs from the bone marrow, DPSCs from both compartments and mixed DPSCs were analyzed for CD29 (BD Biosciences Pharmingen, Belgium), CD34 (Dako, Denmark), CD44 (BD Biosciences Pharmingen, Belgium), CD45 (Dako, Denmark), CD71 (Dako, Denmark), CD90 (BD Biosciences Pharmingen, Belgium), HLA I (Dako, Denmark), HLA II (Dako, Denmark). For karyotyping cells (subcultured at a 1 : 3 dilution, both early passages and after reaching Hayflick's limit) were subjected to a 4-hour Demecolcemid (Sigma, USA) incubation after 24 hours cultivation followed by trypsin-EDTA detachment and lysis with hypotonic KCl and fixation in acid/alcohol. Metaphases were analyzed after GTG banding using software Ikaros v5.0 (MetaSystems, USA).

All experiments were carried out in duplicates, and the results were presented as means and SEMs (standard error of the mean).

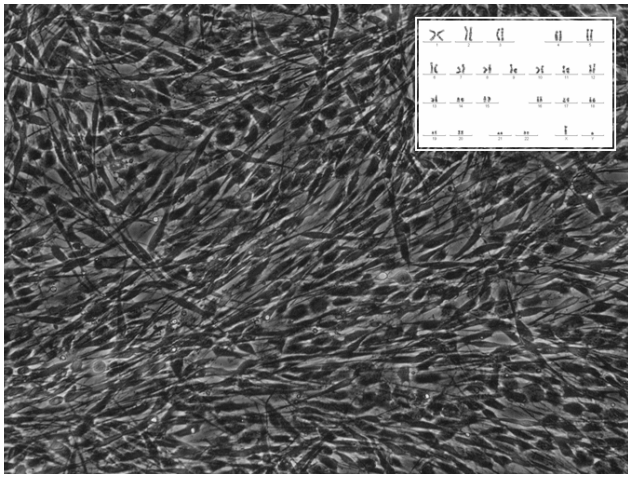
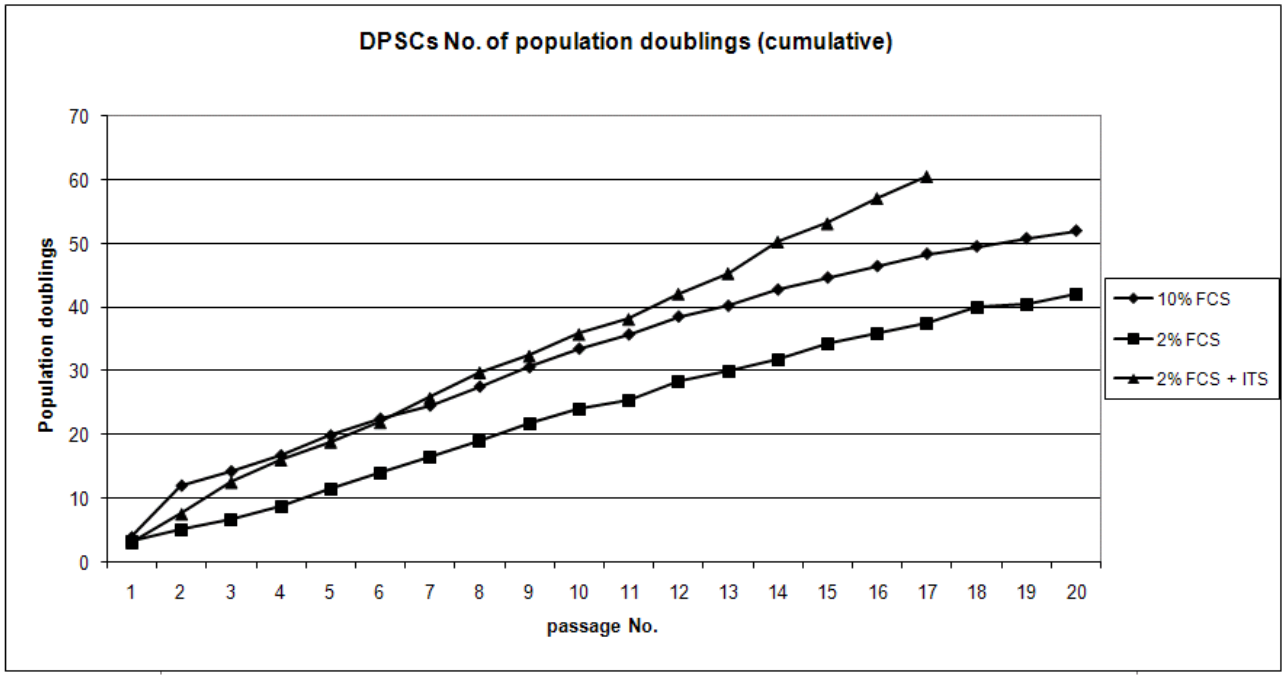


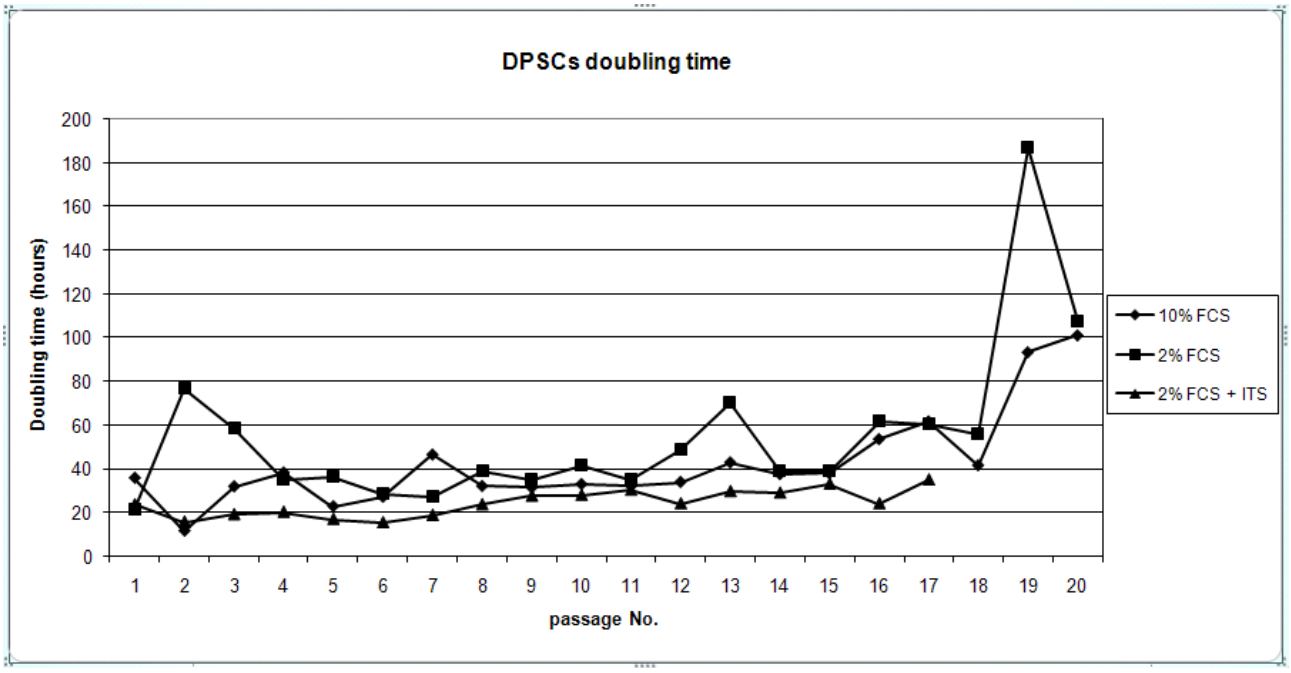
**Fig. 1.** Spindle shaped DPSCs cultivated in 2% FCS with ITS medium after reaching Hayflick's limit. Phase contrast microscopy, DPSCs diameter was 14.25-16.13 µm. Karyotype 46 XY.

## RESULTS

We were able to isolate putative DPSCs and cultivated (Fig. 1) them in three different media (labelled 10 % FCS, 2 % FCS and 2 % FCS with ITS). We obtained an average 46 ± 6 (10–108) DPSCs using enzymatic dissociation of the dental pulp. Primary cultures of DPSCs were inoculated on treated Cell$^+$ surface into different cultivation media. Non-adherent cells and the remnants of pulp tissue




**Graph 1.** Dental pulp stem cells number of population doublings (cumulative).

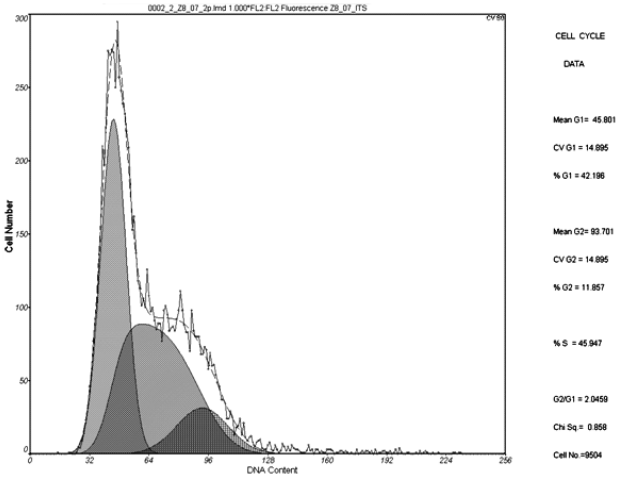



**Graph 2.** Dental pulp stem cells doubling time trend.

were washed down using PBS after 24 hours of inoculation. After 24 hours of cultivation, we observed the first DPSCs, as single cells or as small colonies. After 5 days, we found larger colonies in primary culture and cells were ready for first passaging. Each following passaging was done after reaching 70 % confluence.

We were able to isolate and cultivate 4 lines of DPSCs in 2 % FCS medium, 4 lines of DPSCs in 10 % FCS medium and 8 lines of DPSCs in 2 % FCS medium with ITS supplement.

We expanded DPSCs in 2 % FCS medium over 42 population doublings (PD) (Graph 1). Average doubling time (DT) was 55.43 hours, DT for first 21 PD was 39.98 (21.57 to 77.29) hours, after reaching 21 PD average DT had increased to 68.06 (35.09 to 187.14) hours (Graph 2). Viability in 9$^{th}$ passage (21.8 PD) was 90 %. Diameter distribution of DPSCs showed the stable pat-




**Graph 3.** Dental pulp stem cells (passage No.2) DNA analysis showed high proliferation activity (58.8 % of cells were in $SG_2$ phase).

tern – predominant population was 12.5-17.06 μm in diameter.

DPSCs cultivated in 10 % FCS medium were expanded over 51 PD (Graph 1.). Average DT was 42.56 hours, DT for first 28 PD was 31.04 (11.9 to 46.7) hours, after reaching 28 PD average DT had increased to 50.24 (31.64 to 101.3) hours (Graph 2). Viability in 9$^{th}$ passage (31.04 PD) was 90 %. Diameter distribution of DPSCs presented stable lay-out – predominant population was 12.2-16.6 μm in diameter.

DPSCs cultivated in 2 % FCS medium supplemented with ITS proliferated over 60 PD (Graph 1). Average DT was 24.5 hours, DT for first 29 PD was 19.33 (15.5 to 23.8) hours, after reaching 29 PD average DT had increased to 29.1 (24.19 to 35.1) hours (Graph 2). Viability in 9$^{th}$ passage (29 PD) was 90.4 %. Diameter distribution of DPSCs showed repeatedly lay-out – predominant population was 14.25-16.13 μm in diameter.

Our phenotypical analysis of DPSCs cultivated in 2 % FCS medium showed high positivity for CD 29 (82.4 %), CD 44 (77.9 %), CD 90 (80.9 %) and HLA I (98.0 %). There were no medium positive CDs in our testing panel. CD 117 (16.4 %) was low positive. CD 34 (1.0 %), CD 45 (5.5 %), CD 71 (7.1 %), and HLA II (0.44 %) were negative.

Similar results were found for the phenotypical analysis of DPSCs cultivated in 10 % FCS. Highly positive were CD 29 (98.8 %), CD 44 (99.6 %), CD 90 (99.1 %) and HLA I (99.8 %). Low positivity was shown only for CD 117 (37.8 %). Positivity under 10 % was found for CD 34 (2.3 %), CD 45 (0.3 %), CD 71 (6.9 %) and HLA II (0.1 %).

Phenotypical analysis of DPSCs cultivated in 2 % FCS with ITS supplement showed high positivity for CD 29 (81.1 %), moderate positivity for CD 44 (62.7 %) and CD 90 (51.1 %). Low positivity was shown for CD 45 (35.0 %), CD 117 (35.4 %) and HLA I (39.2 %). CD 34 (0 %), CD 71 (5.8 %) and HLA II (0.0 %) were found negative.

## DISCUSSION

Various more or less successful trials have been conducted to discover a way of functionally and aesthetically, restoring damaged or lost teeth. The possibility of using stem cells, biological molecules and tissue engineering opens new ways for clinical dentistry. For these purposes, dental pulp is well-delimited from other tissues and separate compartment which could be used as a stem cell source. Re-creation of a histocompatible tooth seems to be a perfect method for fully restoring a functional and aesthetically acceptable tooth arch. However, odontogenesis is very complicated process, which cannot be done without understanding DPSC properties. Further, FCS which is often used in the DPSCs cultivation media, disallows them for transplantation. For this reason we reduced the FCS in cultivation medium. In this study we isolated and cultivated 16 lines of DPSCs in three different media, with different concentrations of FCS and with or without ITS supplement, and compared their basic biological properties and phenotype. Unlike other investigators[13,14] we cultivated undifferentiated DPSCs for a long time, over 40 population doublings (Graph 1). After reaching Hayflick's limit, they still had normal karyotype, without any signs of genetic instability. We examined the DPSC doubling time and regression analysis of uncumulated population doublings showed the close dependence of population doublings on passage number and slow decrease of proliferation potential (Graph 2). The study of You-Young Jo[12], which compared different culture condition for DPSCs, periodontal ligament stem cells, periapical follicle stem cells and mandibular bone marrow stem cells cultivated in media with 10 % or 20 % FCS and with or without ascorbic acid, was revealed as the best medium containing 10 % FCS and 100 μM ascorbic acid. We found that decreasing the volume of FCS from 10 % to 2 % had a negative effect on DPSC properties, rapidly increasing population doubling time and decreasing proliferation activity. The average doubling time of DPSCs cultivated in media with 10 % FCS through the whole cultivation was 42.56 hours and they achieved 52 population doublings. For DPSCs cultivated in medium with 2 % of FCS was 55.43 hours and they were able to reach only 42.13 population doubling. On the other hand adding ITS supplement into medium with 2 % FCS stabilized and markedly decreased population doubling time (average doubling time was 24.51 hours) and greatly increased proliferation ability (Graph 3). DPSCs cultivated in media with 2 % FCS and ITS supplement achieved 60.6 population doublings. For this reason it seems that it is possible to decrease volume of FCS in cultivation medium, but the medium should be enriched by growth factors and ITS supplement. Moreover DPSCs cultivated in medium with 2 % FCS and ITS had better biological properties then those cultivated in a medium with 10 % FCS.

Our phenotypical analysis of DPSCs cultivated in all three media showed high or moderate positivity for mesenchymal stem cell markers (CD 29, CD 44 and CD 90). Negativity or low positivity for CD 45 (recognize leuko-

cyte common antigen, monocytes and T-cell subset)[9] and negativity for CD 34 (hematopoietic stem cell marker) suggests that hematopoietic progenitors are not present within the dental pulp. In our study, DPSCs were negative (0 % – 2.3 %) for CD 34 compared to a study by Laino at al.[15] who found CD 34 positive in more than 10 %. CD 117 which is a useful stem cell marker that interacts with stem cell factor and neural crest precursor[16] showed low positivity. For this reason, it seems that part of the DPSCs population could be derived from the neural crest. DPSCs cultivated in 2 % FCS with ITS showed lower positivity for CD 44, CD 90 and HLA I compared to DPSCs cultivated in 2 % FCS or 10 % FCS media. One possible explanation is that ITS keeps DPSCs more undifferentiated and suppresses expression of mesenchymal stem cells markers and HLA I. Before this can be stated, a wider CD testing panel should be used.

## CONCLUSIONS

We isolated and expanded 16 lines of DPSCs in three different media over 40 population doublings. DPSCs cultivated in all media were cytogenetically stable and showed no signs of spontaneous differentiation. Over the entire cultivation period, we observed no changes in cell viability. Medium containing 2 % FCS supplemented with ITS provided better cultivation conditions for DPSCs (shorter DT and more stable proliferation activity were measured) than other tested media (e.g. most cited 10 % FCS or high FCS concentration media). Decrease in concentration of FCS and adding ITS into media had no negative effects on basics biological characteristics (viability, cell diameter). DPSCs cultivated in 2 % FCS with ITS showed lower positivity mesenchymal stem cell markers and HLA I compared to DPSCs cultivated in 2 % FCS or 10 % FCS media.

## *ACKNOWLEDGMENTS*

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