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Human Dental Pulp Stem Cells: recent findings and current research

Krzysztof Janowicz[1], Paul Mozdziak[2], Artur Bryja[3], Bartosz Kempisty[3,4,5], Marta Dyszkiewicz-Konwińska[3,6]

Abstract

Prevalence of neurodegenerative diseases, most of which are life threatening and incurable, is an increasing clinical problem. To date, studies have demonstrated a superior proliferation rate of dental pulp stem cells (DPSCs) compared to other mesenchymal stem cells in vitro. DPSCs has recently been recognized as a novel treatment strategy for neurodegenerative disease, due to their advanced potential for neurogenic differentiation. The oral cavity has been described as a promising source of dental pulp stem cells. DPSCs are widely used in regenerative dentistry holding alternative capacity for osteogenic differentiation and therefore new promises for tissue and whole tooth regeneration. Dental stem cell banking offers a plentiful source of stem cells representing great potential for cell reprogramming and thus cell therapy. Recently, the association of pulp stem cells with three – dimensional scaffold templates allows for building up naturally derived implants. This review introduces to unique properties of DPSCs and biological factors influencing mineralization, proliferation and differentiation of pulp stem cells. Latest research studies are compared in terms of effectiveness and limitations of techniques for the isolation of pulp stem cells, including the enzymatic digestion and the explant culture methods. Moreover, a short overview of most recent findings and clinical application of DPSCs is proffered including progress of current research and limitations still to be addressed in the nearest future. Finally, the article presents new advances in the area of regenerative dentistry and regenerative medicine, including three dimensional printing and three dimensional analysis, emerged to deepen studies under procedures to replace the non patient specific artificial implants.

Running title: DPSCs - review

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[1]The School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Aberdeen, UK
[2]Physiology Graduate Program, North Carolina State University, Raleigh, NC, USA
[3]Department of Anatomy, Poznan University of Medical Sciences, Poznan, Poland
[4]Department of Histology and Embryology, Poznan University of Medical Sciences, Poznań, Poland
[5]Department of Obstetrics and Gynecology, University Hospital and Masaryk University, Brno, Czech Republic
[6]Department of Biomaterials and Experimental Dentistry, Poznan University of Medical Sciences, Poznań, Poland
*** Correspondence:** mdyszkiewicz@ump.edu.pl
Full list of author information is available at the end of article

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Introduction

Isolation of stem cells allowed rapid development of research concerning process of regeneration, creating further possibilities in clinical trials. During past few decades, stem cell therapy revolutionized the area of personalized medicine, bringing plentiful approaches introduced to replace damaged or necrotic tissues into existence [1]. From the number of resources in the human body serving respectively generation, proliferation and differentiation of new stem cells, dental pulp (DP) containing dental pulp stem cells (DPSCs) are the focus of this manuscript. Constantly evolving investigation carried out on the therapeutic effect of pulp cells did not yet go beyond the field of regenerative dentistry and influences it has on patient specific teeth restoration methods, aiming to replace artificial dental tissues [2]. Apart from the described regenerative potential of dental pulp, discovery of multipotent DPSCs offered an alternative to undergoing cell therapies [3]. Additionally, not only DPSCs have proven superior in terms of regeneration of dental tissue, with other cell types, namely apical dental papilla stem cells (SCAPs), human exfoliated deciduous teeth stem cells (SHEDs) and dental follicle precursor stem cells (DFPCs) showing similar properties [4]. Neural crest derived DPSCs are located in the perien-dothelial niche [5]. Due to large accessibility of this biomaterial, serving as potent source of stem cells, researchers came up with two most prominent methods for pulp extraction: explanting and enzimatic digestion [6]. Nowadays, descriptive characteristics of co–cultured DPSCs are based on flow cytometry immunophenotyping, employing a variety of mesenchymal stem cell markers [7]. Therapeutical future of DPSCs lies within treatment of nerve injuries, genetic disorders (e.g. Crohn's disease) and tumour supressive oncogenes [8]. Therefore, even though the most common clinical practice is orientated on the whole tooth reconstruction, recently, DPSCs were identified as a new treatment strategy for degenerative diseases including retinal degeneration, amyotrophic lateral sclerosis or stroke. In regenerative medicine, apart from cell therapy gaining more renown, pulp cells are being widely explored as a stem cell source for transplants [3]. Lately, bioprinting technology successfully produced three dimensional dentin pulp complex that not only contained vascular networks and cancellous bone, but indeed is patient specific, as opposed to commonly used artificial dental implants [9].

Proliferation, differentiation and mineralization

At the beginning of the millennium little was known about the differentiation capacity of dental pulp stem cells, despite researchers had extensive knowledge of how to stimulate growth of mesenchymal tissue for the sake of promoting tooth morphogenesis [10]. *In vivo* conducted studies have proven odontoblastic differentiation and proliferation of DPSCs to not only be depended on pathogen free conditions and blood supply, but also on inflammation due to exposure to the external environment of oral cavity [11]. Lack of control over inflammation in infected tissues has recently been overcome by stimulation with lipopolysaccharide (LPS), in a study aiming to naturally observe the migration process of DPSCs [12]. Considering differences in mRNA expression of mineralization related genes and repair capacity of differently aging DPSCs (under induced inflammatory conditions), there is a growing dependence of proliferation of younger DPSC on upregulated expression of Toll – like receptor 4 (TLR4). Under low LPS concentration, overexpressed TLR4 activates intracellular NF-κB signalling pathway, mediating effective production of cytokines, activation of innate immunity and proliferation of younger DPSCs, in contrast to the aged ones. Furthermore, not only the proliferation capacity decreased with age under induced inflammation, but more importantly the expression of genes responsible for mineralization [13].

When the inflammation is controlled, factors promoting the function of stem cells need to be considered. As for the expression of foetal dental papilla genes being more explicit comparing with dental pulp genes, experimental evidences has shown that epiregulin (EREG) holds the ability to enhance the non-dental epithelial cells differentiation into fully developed epithelium [14]. Broader specificity of EREG to be exploited in other biological processes includes stimulation of DPSCs via activating the MAPK signalling pathway and c-Jun N – terminal kinase, or alternatively mitogen activated protein kinase. Study initiated by Japanese Department of surgery investigated the usefulness of patient derived pulp cells to modulate disease specific pathological mechanism of a disease. Usually, when disease predisposing gene is identified, it is possible to track the progress of stem cell lineage differentiation through analysis of cells collected from patient, to clarify association between causative mutation and cell development [15]. Authors suggested to repair the main mutation using CRISPR/Cas9 system, which should create negative control for mutated DPSCs and simultaneously allow to track the progress of proliferation in either wild type or mutant lineages.

Not only factors affecting growth, speciation or regeneration capacity of DPSCs need to be studied, but more importantly the movement of pulp cells via cell homing into the root canal of tooth. In general, publications lacked research on cell migration, which significantly affected obtained results, as cells that could not reach their destination were deficient in therapeutic capacity [16]. Ke et al., demonstrated that migration of STRO-1 positive DPSCs can be enhanced by expressed microRNA (miRNA-224-

5p), validated further by RT-PCR, including angiogenesis and proliferation. Not only overexpression of miR-224-5p negatively influenced migration of pulp cells but most significantly revealed itself in the P13K – AKT signalling pathway related to cancer and cell apoptosis, its downregulation resulting in cell rescue. Therefore, microRNAs are potentially studied as biomolecules promoting cell migration and necrosis recovery.

Culture and isolation

Increasing viability and expansion range of DPSCs requires use of mallet (alternatively chisel) and cutback of frictional heat in the first place when isolating dental pulp from a single tooth [17]. First investigation to obtain DPSCs through explant culture of dental pulp resulted in harvesting heterogeneous subpopulation of cells [18]. Explant method (outgrowth) not only reproduce substantial amount of good quality DPSCs but more importantly discriminate between genetic expression, morphology and capacity of newly isolated pulp cells to differentiate [19]. Even though lack of previous studies restrained following prediction, Lizier et al. suggested that satisfying yield of subpopulation is depended on the growth requirements of culture. Although many approaches firmly proved the efficiency of explant method in collecting and cultivating dental pulp cells extracted from premolars (or alternatively deciduous, supernumerary or wisdom teeth), quality and volume of pulp cells opposed optimal co–culture and consequently full restoration of the tissue [20]. Granting all this, improvement of the growth technique by Lizier et al. to overcome dense conditions was expected to cause reduction in differentiation pathway and cell marker expression [21]. However, *in vitro* study by Noda et al. have surprisingly demonstrated higher differentiation potential achieved when cells are densely packed, committing more strongly to osteogenic pathway, simultaneously decreasing proliferation rate due to earlier dedication of cells to certain lineages [20,22]. Reports on enzymatic methods for pulp extraction hold practical implications including non – standardized enzyme concentrations which potentially might induce cell death [23]. Even though, mixture of collagenase and other proteinases aids digestion of the pulp, and if associated with mechanical instruments improves cell dissociation; type of association technique, filtering, medium supplementation and size of the tissue fragments all in total create bias reported in method sections [24]. Although, both methods, outgrowth and enzymatic digestion allow harvesting multipotent lineages of pulp cell, authors favour the latter over the former due to better characteristics of yielded cells [25].

Kawashima et al. modified the methods to elevate number of pulp cells through *in vitro* approaches, but also suggested to watch out for densely cultivated cultures, because tight cell to cell contact leads to activation of integrin signalling, which regulates cell fate and proliferation. Sparse cell conditions are more suitable for hard tissue regeneration to maintain the osteoblast and chondrocytes differentiation potential of DPSCs [20]. Isolated heterozygous cell population contain not only DPSCs, but also other types of cells that adhere to plastic, therefore only CD29, CD44, CD105 and STRO-1 positive cells are selected by fluorescence activating cell sorting (FACS), or alternatively magnetic activated cell sorting (MACS) [20,26]. Single Cell Raman Spectroscopy (SCRM) is the first tool to accurately discriminate and select for best quality DPSCs, improving the efficiency of stem cell therapy [27]. SCRM overcomes the heterogeneity issues and, hence, gets rid of double positive results in *ex vivo* studies, such that bio function and phenotypic differences of DPSCs are distinguished by variability in nucleic acid, lipid and protein compartments. As a consequence, it is possible to separate stem cells by age, proliferation capacity, viability and senescence [13].

As mentioned previously, there is a number of factors affecting the expansion of DPSCs, including loose cell contact, low density seeding and culture conditions [28,29]. Furthermore, establishment of standardized conditions including regular replacement of enzymatic mix of collagenase/dispase and control over serum based culturing conditions or even gene expression, all in total influence the stemness properties of pulp cells and large scale cell expansion [30]. Therefore, latest research is directed towards serum/xeno free culturing method (making it more compatible with cGMP practices rather than regulatory authorities) mainly because of limiting precision of serum culturing method and serum derived transmission of zoonotic agents leading to transplant rejection [31]. Although the serum free media does not protect cells from cytotoxic stimuli or UV light and reduce proliferation ability in excessive cultivation, it promotes large scale expansion of stem cells from the dental pulp [31]. Phenotypic fate of genetically unmodified stem cells is not only depended on the serum conditions, but also on external stimuli, such as serum supplementation [32]. Addition of Neurocult™ NS-A proliferation supplement (Stem Cell Technologies, Germany) provokes formation of neurosphere like dentospheres [33]. Brand new protocols include possibility to grow DPSCs by 3D printing cell constructs, creating new window of opportunity and substitute for traditional method of 2D culture growth in flasks [6].

Tissue regeneration and cell therapy

Along with discovery of DPSCs, their repair ability engaged the attention of scientific world [34]. In recent years, innovative research in the areas of regenerative dentistry is largely based on cellular and molecular protocols, aiming to increase the

survival of restored tissues [35]. Even though main focus was set on DPSCs for regeneration of the entire tooth, current approaches put more emphasis on employing biologically friendly strategies to provoke neurogenic and angiogenic commitment of pulp cells [36]. Up to date, variety of aspects are being considered that may induce or repress therapeutic advances, lineage commitment and tumorigenesis of DPSCs. Adipogenesis and osteogenesis pathways distinguish DPSCs from other pulp cells through mediated overexpression of DNMTB3 and G9a genes, as well as methylation of PTEN and carcinogenesis pathways [8]. Supplementation of naturally derived scaffolds (e.g. hyaluronic acid, cellulose, polysaccharides or amniotic membrane) with resveratrol, growth factors and pulp stem cells, improves their growth and proliferation rate due to downregulation of proinflammatory cytokines and anti-oxidant properties [23,37]. Even though synthetic scaffolds (self-assembling peptide or PLGA) create more space for optimization, natural scaffolds retain the native ability to cooperate with bioactive signalling molecules, aiding local regeneration of periodontal ligament (dental connective tissue) [38]. *In vitro* overexpressed vascular endothelial growth factor (VEGF) enhances DPSCs proliferation and cell migration, at the same time expanding angiogenesis (e.g. dental pulp regeneration provided with vessels) [39].

DPSCs are introduced into the field of stem cell therapy to treat neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), due to therapeutic effect of their neurotrophic factors (VEGF, BDNF or NGF) on spinal and motor neurons [40]. Transplantation of cultured DPSCs, containing brain derived neurotrophic factor and nerve growth factor, naturally stimulated the angiogenesis and neurite outgrowth of cultured dorsal root ganglion neurons [41]. Stem cells from human exfoliated deciduous teeth (SHEDs), through promotion of neurite outgrowth, might be useful in treating diabetic polyneuropathy. However, their therapeutic effect is largely depending on the concentration of angiogenic factors and their association with neurogenic factors [42]. As a consequence of increasing availability of disease models in association with novel biomolecules and biostimulators, DPSCs aids successful treatment of diseases of degenerative nature that are usually devastating to the patient and impossible to cure by current healthcare interventions [43]. Animal studies on rat facial nerve regeneration prove therapeutic effect of human immature dental pulp stem cells secreting neurotrophic factors that promote functional recovery of the facial nerve and to trigger mobilization of neuronal survival [44]. Cell therapy by Wang et al. showed preservation of neuromuscular junction innervation and motor neuron survival by administration (at progressing stages of disease) of containing cytokines and trophic factors DPSCs secretome [40]. Therefore, due to neural crest origin, DPSCs show promise as good alternative to be reprogrammed into stem cells of neural lineages [45]. Subsequent studies demonstrated that the closer the origin of particular stem cells to the progenitor cell, the more specific is the modification towards the progenitor. For example, in treatments of stroke-like conditions, the most promising candidates are resident stem cell of the brain and stem cells of neural lineage origin [46].

Research perspectives

Previously used conventional methods had limited capacity for tissue engineering to produce heterogeneous dental – pulp complex in a patient specific manner [2]. Bearing in mind that cell arrangement, growth factors and biological scaffolds in total promote induced localized proliferation, researchers currently employ the cell bead and cell sheet method in which layer by layer, compact and spongy bone tissue constructs containing vascular networks are printed [9]. Future application of STRO-1+cKit+CD34+ DPSCs is based on their ability to form 3D sphere system, capacity to preserve immunological expression of STRO-1+cKit+CD34+ and expression of late neuronal differentiation markers, but also on maintaining capacity for osteogenic differentiation [47]. Specifically, STRO-1+cKit+FLK-1+CD34+ cells were reported to have the highest growth potential and neurogenic commitment, while STRO-1+cKit+CD34+ alone express significantly more low affin-

TABLE 1 Expression profiles of stem cell markers in dental pulp stem cells

AUTHORS	DPSCS MARKER
[11]	general: STRO-1, CD29, CD44, CD73, CD90, CD105, CD146, CD166, CD271 hematopoietic: CD34 and CD117 stemness: OCT-3/4 and NANOG
[25]	CD13, CD29, CD105, CD146, CD166
[1]	CD90, CD73, CD34
[49]	STRO-1, c-Kit, CD29, CD34, CD44, CD73, CD105, CD146
[47]	STRO-1, c-Kit, CD34
[15]	CD90, CD73, CD34

ity nerve growth factor receptor (LNGFR) [47,48]. Therefore, mainly STRO-1+ DPSCs show generally firmer tendency for neurogenic commitment rather than other pulp cells expressing common mesenchymal markers [49,50].

Efficiency of novel DPSC derived treatments is strongly dependent on other cofactors, with root canal treatment recently improved through establishment of silicate – based root canal sealers that exhibited better mineralization activity compared with conventional resin – based sealers [43]. Taiwanese Department of Dentistry, in association with National Taiwan University, demonstrated the importance of accurately established gap width between struts (GWbS) of 3D scaffolds on induced cellular orientation in the process of neural differentiation of DPSCs [51]. Du et al., obtained experimental evidence for spatial control of dentin tissue regeneration through production of bilayered biomimetic tissue scaffold. The bilayered structure divided into open and closed sides provides alternative topographic cues to influence morphology of DPSCs and control osteogenic differentiation through the YAP signalling pathway [52]. In order to observe cell homing and proliferation, new contrast materials are being developed, such as gold nanoparticles associated with poly L-lysine which allow cells to be tracked during 3D analysis (e.g. micro – CT) of interactions between biological scaffolds and dental pulp stem cells [53].

Conclusions

Differentiation capacity of dental pulp stem cells show good promise of alternative therapeutic strategy to treat neurodegenerative diseases. Different factors need to be taken into consideration that may have either positive or negative influence on the osteogenic and neurogenic differentiation activity of DPSCs. Precise regulation of culturing methods and supplementation of co-factors along with control under synthetic and natural environment, allow to trigger the expansion, stemness properties and co-culture of pulp cells. The variety of cells originating from the pulp tissue is enriched for best quality DPSCs through immunophenotyping and cell sorting technologies.

Nonetheless, the lack of experimental evidence related to the cell migration limit currently prevents full understanding and evaluation of cell therapy efficiency and creates more room for further investigation. In addition, other limitations of cell therapies include neurogenic behaviour of animal models, for example poor facial expression of rats, which prevents adequate evaluation of the treatment progress of facial nerve regeneration. Identification and subsequent genetic overexpression of genes and growth factors related to DPSCs proliferation and interconnection with carcinogenesis pathways still needs to be carried on. Finally, even though in the last few years 3D printing technology created emerging backdrop for regenerative endodontics and 3D analysis of connective pulp tissue, potential interference of DPSCs with biological scaffolds, tracking particles or supplementation of serum are yet to be considered.

Ethical approval

The conducted research is not related to either human or animal use.

Corresponding author
Marta Dyszkiewicz-Konwińska, Department of Biomaterials and Experimental Dentistry, 70 Bukowska St., 60-812 Poznań, Poland and Department of Anatomy, Poznan University of Medical Sciences, 6 Święcickiego St., 60-781 Poznań, Poland, Tel./Fax: +48 61 8546565, e-mail: mdyszkiewicz@ump.edu.pl.

Conflict of interest statement
The authors declare they have no conflict of interest.

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