Original article

Comparative analysis of cytokines and growth factors in the conditioned media of stem cells from the pulp of deciduous, young, and old permanent tooth

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ABSTRACT

Objectives: To compare and analyze the secretome profile of stem cells obtained from the deciduous tooth (SHEDs), young (yDPSCs), and old permanent tooth (oDPSCs).
Methods: All the stem cells were assessed for mesenchymal stem cell markers. The stem cells were differentiated into osteoblasts and chondrocytes using lineage-specific differentiation media. Conditioned media was collected from growing stem cells, and a cytometric bead array was performed to estimate secreted cytokines and growth factor levels by flow cytometry. Gene expresseion levels were assessed by real-time quantitative polymerase chain reaction.
Results: Age did not affect the mesenchymal characteristics of dental stem cells from various age groups. The secretomes of SHEDs and young yDPSCs exhibit more growth factors and lesser pro-inflammatory cytokines than oDPSCs. Osteo and chondrogenic differentiation potential were higher in SHEDs and young yDPSCs than in the oDPSCs. TLR1, TLR2, TLR3 show decreased expression levels with age and TLR5, TLR6 show increased expression with age.
Conclusion: The superior regenerative potential of SHEDs and yDPSCs may be due to the higher growth factors and lower pro-inflammatory cytokine profile.

1. Introduction

Dental stem cells are a peculiar category of stem cells acquired from dental pulp, dental apical papilla, dental follicle, and tissue of periodontal ligament (Gronthos et al., 2002, 2000; Liu et al., 2006). Individually these sources yield an unlike the type of dental stem cell. DPSCs and SHEDs are unique because of their neural crest origin and show a high aptitude for differentiation into multiple lineages with the capability to differentiate into adipocytes, odontoblasts, chondrocytes, osteoblasts, adipocytes, chondrocytes, hepatocytes, neuronal cells, and myocytes (Gronthos et al., 2000; Iezzi et al., 2019a; Iohara et al., 2014; Liu et al., 2006). DPSCs have

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been shown to express specific types of markers such as CD106 and STRO-1 and pluripotency markers such as OCT4 and NANOG that are always expressed by mesenchymal stem cells (MSCs) and embryonic stem cells (ESCs) (Gronthos et al., 2002; Liu et al., 2006). Human DPSCs signify a unique type of stem cells presenting great potential for proliferation, self-renewing capability, and differentiation into multiple lineages (Gronthos et al., 2002, 2000; Iezzi et al., 2019a; Iohara et al., 2014). The rate of proliferation and clonogenic ability of SHEDs were found to be greater than that of stem cells obtained from the permanent tooth (Gronthos et al., 2000; Iohara et al., 2014). The characterization of the cells obtained and subsequent studies showed that SHEDs show positive expression for ESC-related markers such as OCT4, SOX2, and NANOG, and MSC-related markers such as CD44, CD49e, and CD146, whereas they are negative for the expression of hematopoietic markers such as CD34 and CD45 also show negative expression for the immunogenic marker HLA-DR (Gronthos et al., 2000; Iezzi et al., 2019b; Iezzi et al., 2019a; Miura et al., 2003; Patil et al., 2018). Additionally, it was revealed that in SHEDs, the presence of pluripotency markers was higher than that of the stem cells from the permanent tooth (Gronthos et al., 2000).

An age-related decline in the potency of pulp stem cells compromises their reparative ability (Hossain et al., 2017; Huang et al., 2009; Miura et al., 2003; Morsczeck, 2019; Nozu et al., 2018; Zhai et al., 2017). Recent investigations on dental pulp stem cell senescence have also shown that the tissue homeostasis of pulp stem cells diminishes with aging (Fesahat et al., 2014; Johnstone and Parashos, 2015; Mas-Bargues et al., 2017; Ning et al., 2020; Nozu et al., 2018). Another aspect of the reparative potential of stem cells is the paracrine secretion of growth factors and cytokines (Diomede et al., 2017; Gronthos et al., 2002; Johnstone and Parashos, 2015; Liu et al., 2006). Deterioration of the differentiation ability towards hard tissues of the dental complex with age is associated with the secretory factors of the dental pulp cells (Iezzi et al., 2019b; Zhang and Chen, 2018). Several reports have shown that inflammatory cytokines secreted from the dental pulp affect their potential to give rise to odontoblasts (Diomede et al., 2017; Fesahat et al., 2014; Ma et al., 2019). However, it is essential to elucidate the effect of aging on the paracrine factors secreted by these stem cells for their successful therapeutic use in regenerative dentistry. Secretome profile rich in growth factors and anti-inflammatory cytokines could potentially possess a higher regenerative potential which in turn could be affected significantly by the age of the source tooth.

Toll-like receptors (TLRs) play a major part in the immune response by identifying the various molecular patterns exhibited by the pathogens. The signaling by TLRs occurs via specific adaptor molecules such as NF-κB and IRFs that determine the course of the innate immune response (Patil et al., 2018). There are 10 members in the human TLR family. Cell surface houses TLRs 1, 2, 4, 5, 6, and 10. TLR3, 7, 8, 9, 11, 12, and 13 are housed within the endosome (Kawai and Akira, 2010; Kawasaki and Kawai, 2014).

The TLR-1 has specificity for gram-positive bacteria and recognizes its pathogen-associated molecular patterns. TLR1 can also be defined as CD281 (Celhar et al., 2012). A TLR-2-dependent microbicidal activity is elicited by bacterial lipoproteins from macrophages 5 that trigger IL-12 production in macrophages and further determines the adaptive immune response (Rock et al., 1998). TLR-3 can recognize components from damaged cells, such as small interfering RNAs, viral double-stranded RNA (dsRNA), and self-RNAs (Brightbill, 1999; Thoma-Uszynski, 2001). TLR-4 can recognize bacterial lipopolysaccharide (LPS) (24). TLR-5 recognizes bacterial flagellin (Thoma-Uszynski, 2001). Stem cells from the dental environment are present in an environment where they face continuously various types of challenges, TLRs are the essential component of innate immune response so it is very important to

analyze their presence on dental stem cells in an age-dependent way.

In the present study, we attempted to carry out a comparative analysis of the secreted cytokines, growth factors, and TLR1 to TLR5 gene expression levels in the stem cells isolated from the pulp of the deciduous tooth and permanent tooth of the old and younger age group.

2. Materials and methods

2.1. Culture and expansion of human DPSCs

Isolated Human dental stem cells were obtained from Regenerative Medicine Laboratory, Dr. D. Y. Patil Dental College & Hospital, Pimpri, Pune – 411018, India (8–12 years old for SHEDs, 14–28 years old for yDPSCs, 50–75 years old for oDPSCs). The DPSCs were further expanded in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and antibiotic–antimycotic solution at 37 °C and 5% CO2. The cell growth media was replaced with fresh media every 2–3 days. At 70–80% confluence cells were detached using 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) solution and expanded further. The passage 4–8 cells were used for all the experimental procedures.

3. Characterization using flow cytometry

For flow cytometry analysis, all three stem cell types were removed and a phosphate-buffered saline (PBS) wash was given to the cells. After incubation with monoclonal anti-CD44-PE, anti-CD73-FITC, and anti-HLA-DR-APC (Miltenyi) antibodies for 30 min at 4 °C, the cells were washed with PBS again and subjected to a flow cytometric analysis (Attune NxT, Thermo Scientific, USA). At least 10,000 events were acquired for each sample. In comparison with the isotype control (FITC and APC), the degree of positively stained cells was calculated for each marker.

4. Differentiation

4.1. Osteogenic differentiation

Cells were incubated for 24 h after seeding at a density of 5000/cm^2 in a 12-well plate. Osteogenic induction media (DMEM with 10% FBS, 10 mM of β-glycerophosphate, 0.1 μM of dexamethasone, and 50 μM of ascorbate-2-phosphate) was introduced to the cells.

The cells were fed with the induction medium every two days. For the control group, the cells were grown on the plain growth medium alone. To check the differentiation into osteoblasts and assess the mineralization, the cells were fixed and von Kossa staining (silver nitrate) was performed after 21 days. The mineralization was quantified by dissolving stained cells in 4% acetic acid, and the absorbance was measured colorimetrically at 450 nm.

4.2. Chondrogenic differentiation

Cells were incubated for 24 h after seeding at a density of 5000/cm^2 in a 12-well plate. Chondrogenic induction media (DMEM with ITS 1X, sodium pyruvate 1 mM, dexamethasone 100 nM, ascorbate-2-phosphate 50 μg/ml, TGF-β3 10 ng/ml, and L-proline 40 μg/ml) was introduced to the cells. The cells were fed with the induction medium every two days. For the control group, the cells were grown on the plain growth medium alone. To check the chondrogenic differentiation and assess the glycosaminoglycan (GAGs) content, the cells were fixed and stained by using 2% alcian blue after 28 days. The GAGs were quantified by dissolving stained

cells in 4% acetic acid, and the absorbance was measured colorimetrically at 630 nm.

4.3. Cytometric bead array for the detection of cytokines and growth factors

A cytometric bead array was performed to determine the level of cytokines and growth factors in the conditioned media. LEGENDplex™ Human Growth Factor Panel (13-plex) (Biolegend; 740180) (Ang-2, EGF, VEGF, bFGF, HGF, SCF, EPO, G-CSF, M−CSF, GM-CSF, PDGF-AA, TGF-α, and PDGF-BB) was used for the detection of growth factors. LEGENDplex™ Human Essential Immune Response Panel (13-plex) (Biolegend;740929) (ILs 1 β, 2, 4, 6, 10, 12p70, 17A, CXCLs 8, 10, CCL2, IFN-γ, TGF-β1, TNF-α) was used for the detection of cytokines. The further experimental protocol was performed conferring to the manufacturer's guidelines. Briefly, 25 μL of the conditioned media was incubated with the microbeads, and detection antibodies were introduced after the tests. The samples were then acquired on the flow cytometer. LEGENDplex™ Data Analysis Software was employed to analyze the data.

4.4. Real-time PCR for analysis of gene expression of TLR –

Total RNA was isolated from all the age group stem cells, RNA was converted into cDNA using a cDNA synthesis kit (Thermofisher). Quantitative gene expression analysis was done for TLR1 (Forward 5′GCCCAAGGAAAAGAGCAAAC and reverse 5′ AAGCAGCAATATCAACAGGAG), TLR2 (Forward 5′ TCTCCCATT TCCGTCTTTTT and reverse 5′GGTCTTGGTGTTCATT ATCTTC), TLR3 (5′ TAAACTGAACCATGCACTCT and reverse 5′ TATGACGAAAGGCACCTATC), TLR4 (5′ GAAGCTGGTGGCTGTGGA and reverse 5′ GATGTAGAACCCGCAAG) and TLR5 (TTGCTCAAA-CACCTGGACAC and reverse 5′ CTGCTCACAAGACAAACGAT) using SYBR Green chemistry on a qRT-PCR machine. Data normalization was done using GAPDH as a reference gene and by using the DeltaCt method data was analyzed.

5. Data analysis and statistics:

The data obtained were designated as the means ± standard deviations of the three independent experimental values. Statistics of the data was performed by paired t-test on GraphPad Prism 8 software. $p < 0.05$ was measured as statistically significant ($^*p < 0.05$ and $^{**}p < 0.01$).

6. Results

6.1. Stem cells from all three sources showed the same degree of positive expression for MSC-specific markers

The stem cells from all three types of sources were positive for both CD44 and CD73 indicating their specificity towards mesenchymal stem cell lineage (Fig. 1). Also, the stem cells from all three types of sources were negative for HLA-DR, a class II MHC surface molecule responsible for any immune response (Fig. 1). Supplementary file contain the raw files.

6.2. Osteogenic and chondrogenic differentiation potentials are affected by the age of the donor

All three types of stem cells showed the aptitude to differentiate towards the osteoblastic and chondrogenic lineages (Fig. 2A). Functional staining aided in eliciting cellular differentiation (Fig. 2A). After quantification of the stained differentiated cells, it was
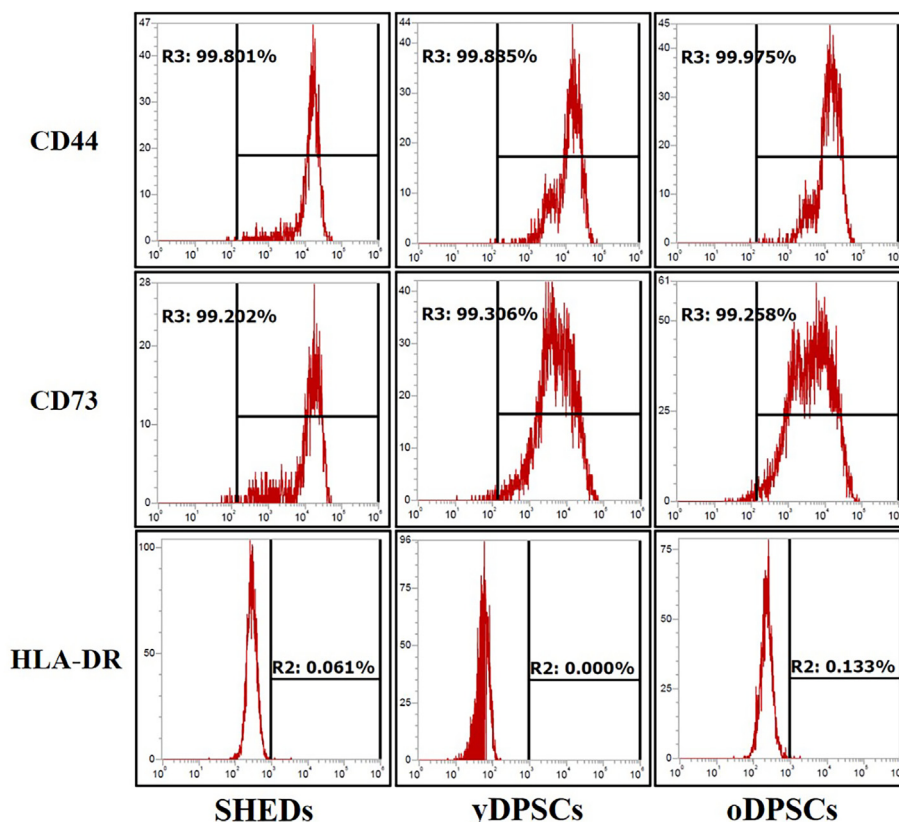

Fig. 1. Flow cytometry analysis of MSC-specific cell surface markers. SHEDs: stem cells from the exfoliated deciduous tooth; yDPSCs: dental pulp stem cells from young donors; oDPSCs: dental pulp stem cells from aged donors.
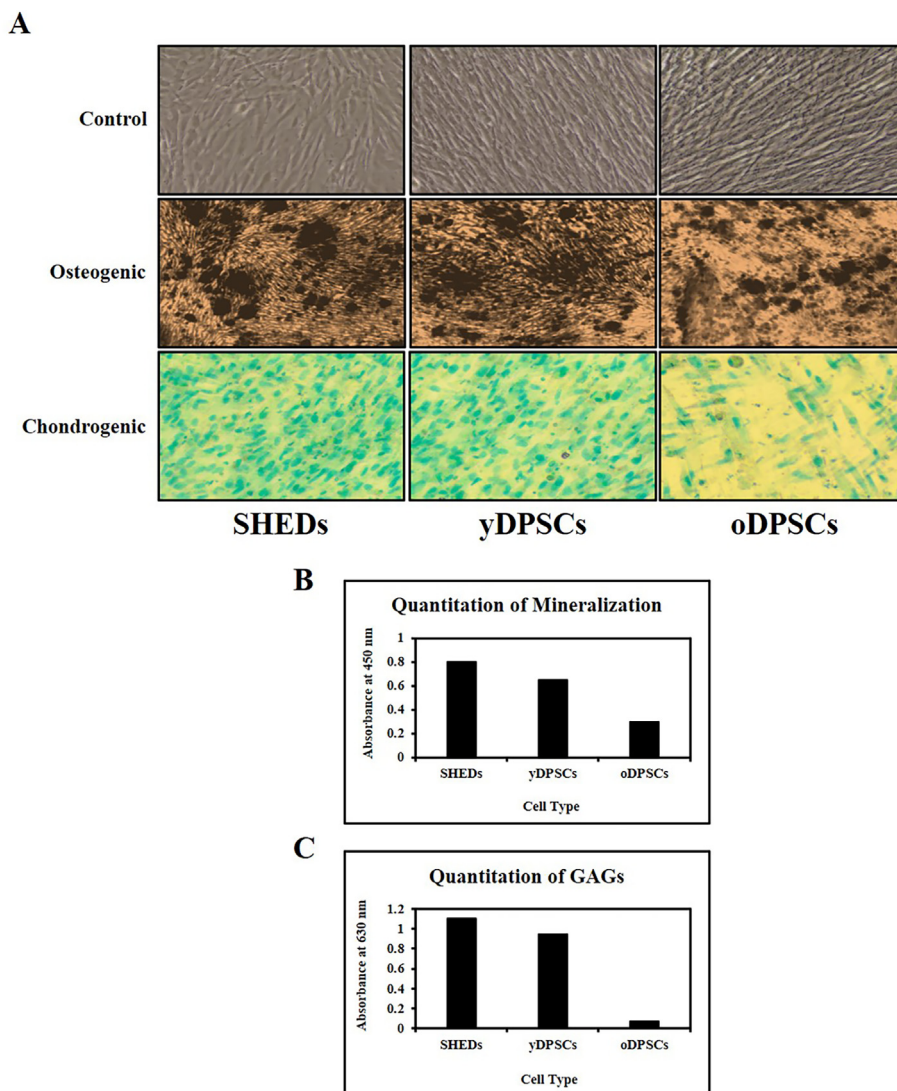
Fig. 2. Differentiation of SHEDs yDPSCs and oDPSCs into osteoblasts and chondrocytes and quantification of mineralization and GAGs. **(A)** Osteogenic differentiation stained with von Kossa method and chondrogenic differentiation stained with alcian blue. Scale bar = 100 µm. **(B)** Quantitation of mineralization in von Kossa staining. **(C)** Quantitation of GAGs in alcian blue staining. SHEDs: stem cells from the exfoliated deciduous tooth; yDPSCs: dental pulp stem cells from young donors; oDPSCs: dental pulp stem cells from aged donors; GAGs: glycosaminoglycans.

revealed that the potential for differentiation in osteo/chondrogenic lineages was affected in the aged permanent tooth in comparison with that from the deciduous tooth and young permanent tooth (Fig. 2B and C).

6.3. Stem cells from the deciduous tooth show higher levels of secretary growth factors and low levels of proinflammatory cytokines

The analyses of secretomes of all three types of stem cells show that the stem cells from the aged tooth are not as efficient as stem cells from the young permanent tooth and deciduous tooth in the secretion of growth factors. Additionally, stem cells from the aged tooth secrete proinflammatory cytokines in higher levels than stem cells from the young permanent and deciduous tooth (Fig. 3A). Stem cells from deciduous teeth are superior in the secretion of growth factors (Fig. 3B).

6.4. SHEDs, yDPSCs, and oDPSCs show variable expression of TLR genes

The comparative analysis of three age group stem cells concerning TLR genes revealed a remarkably interesting pattern. SHEDs

and yDPSCs showed higher expression of TLR1-3 and oDPSCs showed lower levels of gene expression for the same. The situation was interestingly got inversed in the case of TLR-4 and TLR-5, where oDPSCs got the upper hand concerning gene expression (Fig. 4).

7. Discussion

Aging causes a decline in all tissues and organs (Hossain et al., 2017; Iezzi et al., 2019a; Ning et al., 2020; Patil et al., 2018), and the dental pulp is no exception to this phenomenon. Numerous reports are stating that the reparative potential and defense mechanisms of the dental pulp are compromised due to aging (Hossain et al., 2017; Kawasaki and Kawai, 2014; Miura et al., 2003; Zhai et al., 2017). Aging not only affects the plasticity of the dental pulp stem cells but also contributes to extracellular matrix production (Ma et al., 2019; Zhang and Chen, 2018). The secretion of paracrine factors (Kawai and Akira, 2010; Ma et al., 2009; Nozu et al., 2018) is also thwarted by aging, which causes the odontoblasts to lose their potency to form dentin (Celhar et al., 2012; Rock et al., 1998).
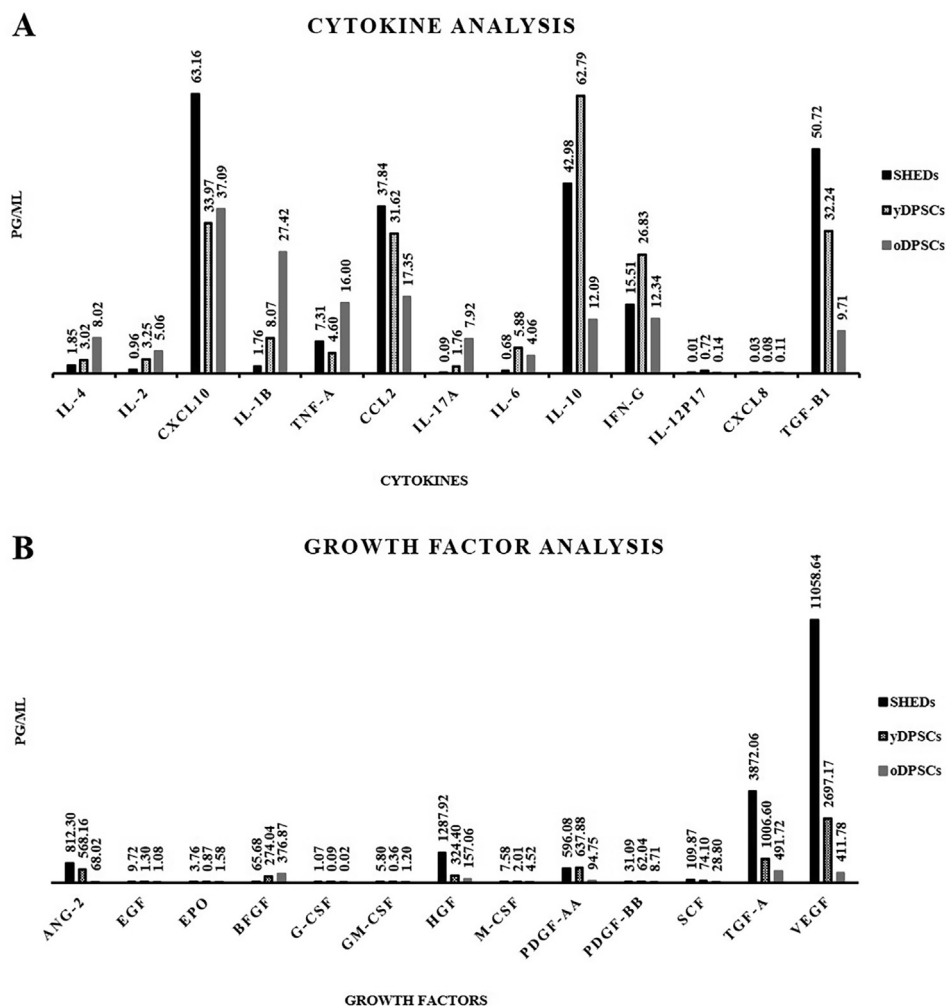
Fig. 3. Cytokine and growth factor analysis of conditioned media from SHEDs yDPSCs and oDPSCs. **(A)** Comparative analysis of cytokine secretion in conditioned media from SHEDs yDPSCs and oDPSCs. **(B)** Comparative analysis of growth factor secretion in conditioned media from SHEDs yDPSCs and oDPSCs. SHEDs: stem cells from the exfoliated deciduous tooth; yDPSCs: dental pulp stem cells from young donors; oDPSCs: dental pulp stem cells from aged donors.
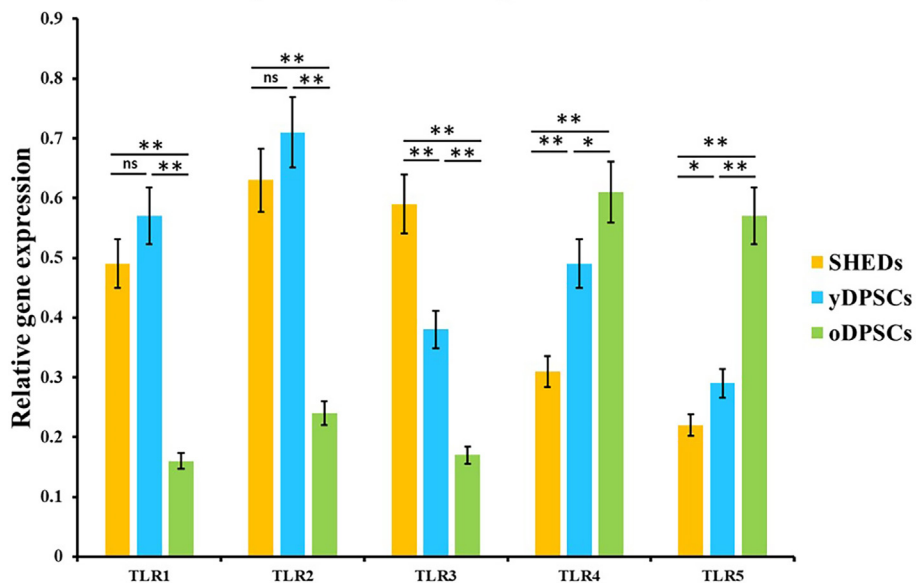

Fig. 4. Gene expression analysis by RT-qPCR. Comparative analysis of TLR1, TLR2, TLR3, TLR4, and TLR5 in SHEDs, yDPSCs, and oDPSCs. SHEDs: stem cells from exfoliated deciduous tooth; yDPSCs: dental pulp stem cells from young donors; oDPSCs: dental pulp stem cells from aged donors.

In our study, we found that there was not much difference in MSC markers in all three types of stem cells. However, assessing their potential to differentiate into osteoblasts and chondrocytes revealed that aging severely affects the matrix formation by stem cells. Stem cells are the primary source for maintaining both hard and soft tissue homeostasis. An inferior matrix-forming ability of stem cells from the aged tooth indicates its attenuating regenerative potential limiting its application in modulating osteintegration for dental implants, alveolar bone augmentation, and rehabilitation of the periodontium.

We further collected the conditioned media from all three types of stem cells. The secretome of SHEDs showed higher levels of the growth factors Angiopoietin-2, VEGF, EGF, HGF, SCF, and TGF-α. The stem cells from aged pulp showed very poor growth factor secretions, and the growth factors in the secretome of adult pulp stem cells were also less than that of deciduous tooth pulp stem cells. In the cytokine analysis of the secretomes, it was evident that the pro-inflammatory cytokines (ILs-6, 1β, TNF-α, IFN-γ) were secreted highly in the conditioned media of yDPSCs and oDPSCs than that of SHEDs. On the other hand, the secretome of SHEDs had a greater expression of TGF-β1 and IL-10 (anti-inflammatory cytokines). The chemokine receptor-ligand CXCL10 and CCL2 were also higher in the conditioned media of SHEDs. When we analyzed the gene expression of TLRs, the essential components of the innate immune response, it was observed that TLR-1, TLR-2, and TLR-3 selectively expressing higher in the stem cells from the younger age group, on the other hand, and TLR-4 and TLR-5 were expressing on a higher side in the stem cells from old age group.

Aging has caused a predominantly pro-inflammatory cytokine profile, with a declining growth factor profile. Although the reduced growth factor profile attenuates the regenerative potential, the pro-inflammatory profile is the larger concern as it can augment the host's immune response potentially causing adverse inflammatory reactions. Thus, the secretome of all isolated and characterized stem cells must be profiled for cytokine and growth factors to determine their regenerative and inflammatory potential before utilizing them for any application. Future studies could overcome the effects of aging by augmenting the growth factor profile or by attenuating the pro-inflammatory cytokine profiles through conditioning. A tailored secretome could provide optimal regeneration without any significant potential for an adverse reaction.

In the field of dentistry, very little is known about aging and senescence and the extent to which it affects the normal reparative mechanisms of the oral tissues. Most studies have focused only on senescence-associated secretory factors and telomere attrition (Brightbill, 1999; Rock et al., 1998; Thoma-Uszynski, 2001). Also, understanding the underlying mechanisms of the secretion of the stem cell and its potential applications in tissue regeneration would help to further optimize their potency in the field of regenerative dentistry. The present study provides an overview of secretory factors by the pulp stem cells of the different age groups at a glance as well as puts a light on TLR expression. However, it is essential to study the complex signaling processes invoked by these signaling molecules and receptors to understand the regenerative and immune biology of these cells.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.03.031.

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