APPLICATION OF DENTAL MESENCHYMAL STEM CELLS FOR CHARACTERIZATION OF OSTEOGENIC DIFFERENTIATION AND FOR CITOTOXICITY STUDY ON MOUTHWASHES

PhD thesis

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LIST OF ABBREVIATIONS

αMEM	alpha modification of minimum essential medium Eagle
ALPL	alkaline phosphatase gene
BGLAP	gene of bone gamma-carboxyglutamic acid-containing protein
BSP	bone sialoprotein
CD105	endoglin
CD73	ecto-5′-nucleotidase
CD90	Thy-1 (thymocyte differentiation antigen 1)
CEM-1/CEMP-1	cementum protein-1
CHX	chlorhexidine
ClO_2	chlorine dioxide
DFSC	dental follicle stem cell
DMEM	Dulbecco's modified Eagle medium
DMSC	dental mesenchymal stem cell
DPSC	dental pulp stem cell
DSC	dental stem cell
FBS	fetal bovine serum
FGF-2	fibroblast growth factor-2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H_2O_2	hydrogen peroxide
IBSP	bone sialoproteine gene
iPS	induced pluripotent stem cells
MEM	minimal essential medium
MSC	mesenchymal stem cell
NEAA	non-essential amino acids solution
NFIC	nuclear factor I-C
OC	osteocalcin
ON	osteonectin
Osx	osterix
PBS	phosphate buffered saline
PDLSC	periodontal ligament stem cell

PLAP-1	periodontal ligament-associated protein-1
RQ	relative quantification
RT	room temperature
RT-qPCR	real-time-quantitative polymerase chain reaction
Runx2	runt-related transcription factor 2
SCAP	stem cells from the apical papilla
SHED	stem cells from human exfoliated deciduous teeth
SP7	osterix gene
STRO-1	first antigene identified on stromal cells of bone marrow

1. INTRODUCTION

1.1. Dental mesenchymal stem cells

One of the most intensively developing areas of modern medicine is stem cell research, including dental stem cell research. Dental stem cells (DSCs) have the advantages of being easily available and having multipotent differentiation ability, thus promising therapeutic potential beyond tooth regeneration to the regeneration of other tissue types *(Chalisserry et al, 2017, Dave and Tomar 2018)*. Dental pulp stem cells (DPSCs) were the first dental stem cells to be isolated from the pulp of permanent teeth *(Gronthos et al, 2000)*. Later, the same research group isolated stem cells from human exfoliated deciduous teeth (SHED) *(Miura et al, 2003)*. Seo et al. were the first to isolate periodontal ligament-derived stem cells (PDLSC) from extracted human third molars *(Seo et al, 2004)*. In the following year, it was described for the first time that stem cells could be isolated even from the dental follicle of wisdom teeth *(Morsczeck,Gotz et al, 2005)*. Then, in 2006, the fifth dental stem cell type was isolated from the apical papilla (SCAP) *(Sonoyama et al, 2006)* (Figure 1).

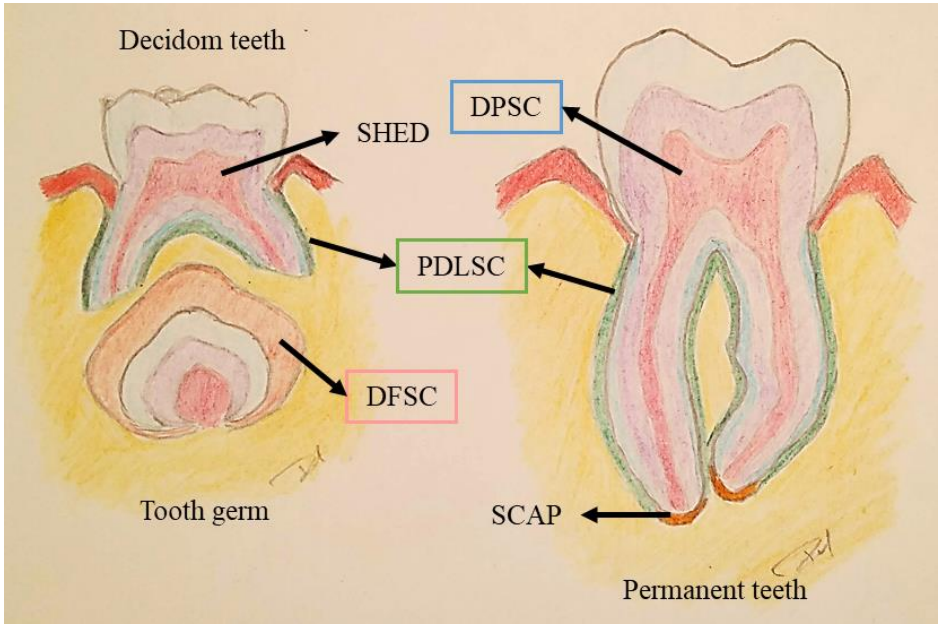

Figure 1

Schematic illustration of the location and origin of dental stem cells (*Illustration by the author).*

All of the aforementioned dental mesenchymal stem cell (DMSC) types are able to differentiate into osteogenic, adipogenic, chondrogenic, and myogenic lineages *in vitro (Huang et al, 2009)* while they can take part in the regeneration of different dental tissues (including dentin, pulp, cementum, and periodontal ligament) *in vivo (Huang et al, 2009, Shuai et al, 2018).* Moreover, several literature data underline that these can be differentiated even into neuronal phenotypes *in vitro (Huang et al, 2009, Kadar et al, 2009, Kiraly et al, 2009)* as a consequence of the fact that teeth originate from the neural crest. Based on *in vivo* experiments, DMSCs are suitable for application not only in the field of regenerative dentistry *(Tatullo et al, 2019)* but also in bone tissue engineering *(Yusof et al, 2018)* and even in stem cell-based therapies of neurological diseases *(Relano-Gines et al, 2019).* Several pieces of evidence confirm the immunomodulatory effect of dental stem cells *(Kang et al, 2015, Foldes et al, 2016, Whiting et al, 2018),* which is a further favourable feature of these cells in clinical use.

1.1.1. Dental pulp stem cell (DPSC) and stem cells from human exfoliated deciduous teeth (SHED)

The innermost layer of teeth is the pulp – a network of mesodermal tissue, loose fibrous connective tissue, capillaries, and nerves. Its main functions are to transmit heat, pain, and tactile stimuli to the brain and to respond to tooth-damaging effects through inflammatory reaction and formation of tertiary dentin.

It has been proven for more than twenty years that stem cells are present in teeth as well *(Gronthos et al, 2000)* (Figure 1), and that these stem cells can be obtained by several methods. Both explant cultures and enzymatic digestion methods can be similarly effective in isolating DPSCs *(Hilkens et al, 2013).*

DPSCs show a fibroblast-like morphology *(Dave and Tomar 2018).* Several protein markers have been detected in DPSCs, including CD13, CD29, CD34, CD44, CD90, CD105, CD146, STRO-1 *(Huang et al, 2009, Suchanek et al, 2009, Bakopoulou et al, 2011, Dave and Tomar 2018),* vimentin, and nestin *(Karbanova et al, 2011).*

It has been described that DPSCs can differentiate in several directions, including osteogenic *(d'Aquino et al, 2007, Huang et al, 2009, Kadar et al, 2009, Kraft et al,*

2010, Del Angel-Mosqueda et al, 2015, Noda et al, 2019) neurogenic *(Huang et al, 2009, Kiraly et al, 2009, Madanagopal et al, 2020),* chondrogenic *(Huang et al, 2009, Lei et al, 2014),* adipogenic, myogenic lineages *(Huang et al, 2009),* and even into vascular endotheliocytes *(d'Aquino et al, 2007, Li et al, 2020).* It is of particular interest that osteoblasts and endotheliocytes are simultaneously formed from DPSCs during bone regeneration, creating new bone tissue with vascular supply *(d'Aquino et al, 2007).*

DPSCs can be isolated not only from extracted third molars, but also from extracted first premolars *(d'Aquino et al, 2007, Mehrabani et al, 2017).* Tooth pulp is available not only in permanent teeth, but can also be isolated from deciduous teeth *(Miura et al, 2003)* (Figure 1). Several studies compare these two cell types. Deciduous tooth-derived stem cells have higher proliferation and differentiation potential, while stem cells from permanent teeth show higher inflammatory cytokine levels *(Kunimatsu et al, 2018).* Flow cytometry was used by Wang et al. to compare CD73 +, CD90 +, and CD105 + cells in low (P4) and high (P20) passages, where it was shown that SHED is markedly more differentiable in P4 than in P20 passage *(Wang et al, 2018).*

Another important feature is that DPSC from the extracted teeth of adults aged 30 to 45 can also be differentiated into the osteogenic direction *(Laino et al, 2005),* which suggests that DPSC is a very easily accessible cell source for stem cell therapy in this age group.

SHED has also been reported to be positive for CD44, CD73, CD90 *(Zhang et al, 2016),* CD105, CD166 *(Yamaza et al, 2010),* CD143, and STRO-1 markers *(Miura et al, 2003).* This cell type can be differentiated into osteogenic *(Miura et al, 2003, Zhang et al, 2016),* adipogenic, neurogenic *(Zhang et al, 2016)* and hepatogenic *(Yamaza et al, 2015)* lineages.

1.1.2. Periodontal ligament stem cell (PDLSC)

Periodontal ligament is a connective tissue, rich in cells and collagen fibres, that fills the 0.18-0.25 mm wide space between the cement and the wall of the alveolar bone.

Periodontal ligament stem cells can be isolated not only from residual teeth (third molar teeth or premolar teeth extracted due to orthodontic treatments) but also from

deciduous teeth *(Silverio et al, 2010)* (Figure 1). Comparing PDLSCs from deciduous teeth and those from permanent teeth, the former have a higher proliferation rate and colony-forming capacity. Moreover, ALP activity, degree of mineralization, and expression rate of mineralization-related genes are significantly higher during osteogenic differentiation of deciduous tooth PDLSCs *(Ji et al, 2013).*

PDLSCs show a fibroblast-like morphology, forming flat and loose aggregates in cell cultures *(Kawanabe et al, 2010).* PDLSCs are similar to DPSCs in terms of surface markers; they are also positive for CD13, CD44, CD73, CD90, CD105, nestin, BSP, etc., and negative for CD19, CD34, CD45, etc. *(Trubiani et al, 2005, Coura et al, 2008, Huang et al, 2009, Ding et al, 2010, Iwasaki et al, 2013).*

PDLSCs are differentiable into the osteogenic *(Seo et al, 2004, Nagatomo et al, 2006, Lin et al, 2008, Kim et al, 2013, Tang et al, 2014, Nagy et al, 2018, Li et al, 2019),* neurogenic *(Coura et al, 2008, Tomokiyo et al, 2008),* myogenic *(Song et al, 2012)* adipogenic *(Seo et al, 2004),* cementogenic and chondrogenic directions *(Seo et al, 2004, Maeda et al, 2011, Liu et al, 2015).*

PDLSCs can also be used in several fields of regenerative medicine *(Su et al, 2015).* For example, Chen et al. studied the effect of PDLSCs on bone regeneration in a surgical therapy of periodontitis with bone defect. They have shown in this forward-looking follow-up study that PDLSCs also significantly improve bone defect *(Chen et al, 2016).* The importance of PDLSCs in regenerative medicine is also supported by the successful production of PDLSCs from induced pluripotent stem (iPS) cells *(Hamano et al, 2018),* giving an alternative to extracted teeth in order to gain PDLSCs.

The significant regenerative capacity of PDLSCs allows the periodontal region to heal and provides the opportunity for it to be involved in restorative mechanisms *(Su et al, 2015, Kammerer et al, 2017).* The differentiation potential of these stem cells is similar to that of pericytes, while their immunomodulatory nature through inhibiting T cell proliferation has been well described *(Ding et al, 2010, Iwasaki et al, 2013).*

These favorable properties played a role in our choosing PDLSCs from the three dental stem cell types for our toxicological study with mouthwashes.

1.1.3. Dental follicle stem cell (DFSC)

The dental follicle is a capsule-like formation of mesodermal origin in which the tooth is developing. Later it forms the cement, the periodontal ligament system, and the alveolar bone surrounding the teeth. Like other dental stem cells, DFSCs also show a fibroblast-like morphology *(Dave and Tomar 2018)* and can be obtained from ectopic impacted teeth or impacted third molars *(Shuai et al, 2018)* (Figure 1).

Dental follicle stem cells express several protein markers, including STRO-1, vimentin, CD29, CD146 *(Guo et al, 2013)*, CD44, CD90 *(Guo et al, 2013, Sowmya et al, 2015)*, CD73 *(Sowmya et al, 2015)*, and CD105 *(Dave and Tomar 2018)*. To demonstrate that DFSCs have the potential to form periodontal ligament, fibroblasts, or cementoblasts, Sowmya et al. described that DFSCs are positive for periodontal ligament-associated protein-1 (PLAP-1), fibroblast growth factor-2 (FGF-2), and cementum protein-1 (CEMP-1) markers *(Sowmya et al, 2015)*.

DFSCs are also a multipotent stem cell type. These cells can be differentiated in several directions, including osteoblasts *(Morsczeck, Moehl et al, 2005, Guo et al, 2013)*, cementoblasts *(Yao et al, 2008, Sowmya et al, 2015)*, adipocytes, neurons *(Yao et al, 2008)*, and fibroblasts *(Sowmya et al, 2015)*. It is interesting to note that Felthaus et al. demonstrated that osteogenic differentiation of DFSCs is independent from Runx2 *(Felthaus et al, 2014)*.

However, results contrary to the above can also be found in the literature. Liu et al. investigated the effect of Runx2 gene manipulation on osteogenic differentiation of DFSCs. They found that the Runx2 mutation may reduce the ability of DFSCs to differentiate in the osteogenic direction, but may increase their ability to proliferate compared to the wild (non-mutated gene) type *(Liu et al, 2019)*.

When frozen and freshly isolated cells were compared by Kang's research group, no significant difference was found in respect to new bone formation activities. Therefore, dental stem cells may be suitable for either autologous or allogeneic stem cell therapy or tissue engineering purposes even after freezing *(Kang et al, 2015)*.

Comparing dental follicle stem cells with dental papilla stem cells, it was demonstrated that although both have strong proliferative capacity, DFSCs have higher proliferation rates and telomerase activity *(Guo et al, 2013)*. Regarding their ability to

differentiate in the osteogenic and adipogenic directions, DFSCs differentiate to a greater extent in 25 days in both directions than DPSCs *(Guo et al, 2013).*

1.1.4. Stem cells from the apical papilla (SCAP)

Stem cells from the apical papilla can be isolated from the cell cluster at the root apex of developing teeth *(Dave and Tomar 2018).* This cell cluster is involved in root development, and the formation of dentin and cement *(Yu et al, 2016).* These stem cells show a fibroblast-like morphology *(Liu et al, 2015).*

Among the protein markers expressed on SCAPs are STRO-1 *(Bakopoulou et al, 2011, Bakopoulou et al, 2013),* CD34 *(Bakopoulou et al, 2011, Zhang et al, 2015),* CD73 *(Yu et al, 2016),* CD90, CD105 *(Zhang et al, 2015),* CD 143 *(Bakopoulou et al, 2013),* and CD146 *(Bakopoulou et al, 2011, Yu et al, 2016).*

Stem cells from the apical papilla can be differentiated into adipogenic, osteogenic *(Zhang et al, 2014),* osteo/odontogenic *(Bakopoulou et al, 2011, Zhang et al, 2015)* lineages, hepatocyte-like cells and neural cells *(Dave and Tomar 2018).*

I would like to mention the interesting results published by Zhang W et al. and Zhang J et al. The former research group showed that lentiviral plasmid transfected by the BMP2 gene enhances the odontogenic differentiation of SCAPs *(Zhang et al, 2014).* The latter research group succeeded in increasing the osteo/odontogenic differentiation of SCAPs by upregulation of NFIC (Nuclear Factor IC) transcription factor activity, consequently NFIC plays a role in regulating tooth root development *(Zhang et al, 2015).*

In my dissertation, I examined DFSCs, PDLSCs, and DPSCs during osteogenic differentiation.

1.2. Osteogenic differentiation protocols and studies on dental stem cells

1.2.1. Osteogenic differentiation protocols

All dental stem cells are able to differentiate in the osteogenic direction *(Huang et al, 2009)*. Although several studies have proved the osteogenic differentiation capacity of these cells, a comprehensive study such as ours involving at least three dental stem cell types under the same circumstances and investigating several sorts of cellular and molecular changes can hardly be found in the literature *(Zhang et al, 2018)*. Moreover, it is important to mention that in almost all the studies with dental stem cells a slightly different osteogenic differentiation protocol was used *(Hoemann et al, 2009)*. The basal cell culture medium is typically one from MEM (Minimum Essential Medium Eagle), αMEM (Minimum Essential Medium Eagle – alpha modification) or DMEM (Dulbecco's Modified Eagle Medium), which are usually supplemented with some of the following inductive factors: L-ascorbate 2-phosphate, KH_2PO_4, β-glycerophosphate, dexamethasone, and 1α,25-dihydroxyvitamin D_3 in a changeable concentration. Moreover, the FBS concentration varies from 20% *(Gronthos et al, 2000, Mangano et al, 2010)* through 15% *(Galler et al, 2008, Wei et al, 2008)*, 10% *(Nagatomo et al, 2006, Kawanabe et al, 2010, Kraft et al, 2010, Yan et al, 2014, Rezai-Rad et al, 2015, Goto et al, 2016)* and 5% *(Lin et al, 2008)* to 1% *(Kemoun et al, 2007, Kadar et al, 2009)*. Besides, Prof. Papaccios' research group – applying αMEM that contains ascorbic acid *per se* – it was reported in several papers that FBS (fetal bovine serum) in high concentration (20%) *per se* can induce osteogenic differentiation of DPSCs in long-term experiments *(Laino et al, 2005, d'Aquino et al, 2007, Mangano et al, 2010)*.

As in our case, many research groups induce osteogenic differentiation with a combination of ascorbic acid, dexamethasone, and inorganic phosphate *(Simmons and Torok-Storb 1991, Gronthos et al, 2000, Miura et al, 2003, Seo et al, 2004, Sonoyama et al, 2006, Gay et al, 2007)*. The concentration of dexamethasone is also highly variable, from 10^4 nM *(Silverio et al, 2010)*, 10^2 nM *(Morsczeck, Moehl et al, 2005, Hayami et al, 2007)* to 10 nM *(Hayami et al, 2007, Guo et al, 2013, Felthaus et al, 2014)*. The effect of dexamethasone concentration on PDLSC cells was studied by Kim et al., and it was found that 10^2 nM and 10^3 nM dexamethasone concentrations resulted in the most robust differentiation process *(Kim et al, 2013)*. During the ossification

process, cells produce a mineralised exracellular matrix. The formation of the matrix is influenced by ascorbic acid 2-phosphate, which causes the cells to produce type I collagen. Dexamethasone increases ALP activity, also contributing to matrix formation, but a mineralised matrix requires β-glycerophosphate, which incorporates inorganic salts into the exracellular matrix. If the differentiation medium is αMEM-based, β-glycerophosphate will also incorporate inorganic phosphate into the mineralised matrix (*Hoemann et al 2009*). Therefore, our team added ascorbic acid 2-phosphate, dexamethasone, and β-glycerophosphate to the αMEM-based differentiating solution.

The mineralization of the cells, i.e. calcium deposit formation, is usually confirmed by two staining methods: Alizarin Red staining *(Simmons and Torok-Storb 1991, Gronthos et al, 2000, Miura et al, 2003, Seo et al, 2004, Sonoyama et al, 2006)* or von Kossa staining *(Morsczeck, Gotz et al, 2005, Morsczeck, Moehl et al, 2005, Silverio et al, 2010).* In Alizarin Red staining, the lake pigment causes an orange or red colour reaction in the presence of calcium. In von Kossa staining, the conversion of organic calcium salts into silver salts leads to a brown colour reaction when exposed to light. The two staining methods were carried out in a comparable way, for example, by Deegan et al. *(Deegan et al, 2014).* Their photographs – in accordance with our observations – show that the Alzarin Red dye stains not only the calcium deposits but also causes a slight background staining on the cells. For this reason we have chosen the von Kossa calcium staining procedure. Incidentally, the latter method was invented by Hungarian scientist Gyula Magyary-Kossa in the early 1900s.

1.2.2. Immunphenotype of dental stem cells

Although their advantageous features regarding tissue regeneration have been reported in several studies, the basic cell biological properties of the dental-tissue-derived mesenchymal stem cells (MSCs) are not fully understood. They share many common properties with bone marrow mesenchymal stem cells (BMMSCs) with regard to multipotentiality and *in vitro* phenotypic characteristics *(Huang et al, 2009).* Similarly to BMMSCs, most DSCs express CD73 (ecto-5′-nucleotidase), CD90 (Thy-1), and CD105 (endoglin) cell surface protein markers *(Zhang et al, 2018)* –defined as

one of the minimal criteria for multipotent MSCs *(Dominici et al, 2006)*. In addition, several DSC types express osteogenic markers like osteonectin (ON) and bone sialoprotein (BSP) together with nestin in accordance with BMMSCs *(Huang et al, 2009)*. Nestin is an intermedier filament that was originally described as a marker for neuroepithelial stem cells *(Lendahl et al, 1990)* and has long been regarded as a neural marker of MSCs. Recently, its feasibility became questionable because nestin was detected in odontogenic tumors as well *(Fujita et al, 2006)*, moreover its expression did not change during osteogenic differentiation of BMMSCs *(Wong et al, 2014)* and its participation in angiogenesis has also been reported *(Xie et al, 2015)*. Therefore, further investigations are required to deepen the understanding of the role of nestin in neurogenesis and MSCs.

The STRO-1 antigen can also be detected in cultures of various tooth-derived stem cells *(Huang et al, 2009)*. This antigen was first identified on the surface of human bone marrow stromal cell precursors *(Simmons and Torok-Storb 1991)*. It was later demonstrated that the STRO-1 positive cell fraction of the human bone marrow contains osteogenic precursor cells *(Gronthos et al, 1994)*. Subsequently, it was proven that STRO-1+ bone marrow cells are capable of differentiating into multiple mesenchymal lineages *(Dennis et al, 2002)*, and STRO-1 came to be considered as an MSC marker. Several studies reported the selection of the STRO-1 positive fraction of stem cell cultures derived from bone marrow *(Byers et al, 1999)* or dental tissues *(Yang et al, 2007, Bakopoulou et al, 2013)* by cell sorting. However, it recently turned out that selection for the STRO-1 marker does not enhance the osteogenic capacity of cells *(Yan et al, 2014, Ercal et al, 2017)*. In addition, STRO-1 also proved to be present in immature and mature cartilage *(Otsuki et al, 2010)* as well as in the endothelium or near the vessels in many other organs, including the liver, lungs, kidneys, or skeletal muscle *(Lin et al, 2011, Ning et al, 2011)*. Consequently, the role of the STRO-1 marker in the stemness or osteogenesis of dental stem cells remained to be clarified.

STRO-1+ cells are most commonly tested by flow cytometry, which requires unharmed cells. During the process of ossification, calcium salts are deposited in the extracellular matrix, preventing the cells from being removed from the culture dish intact. This may explain why practically no data can be found in the literature regarding

changes in the proportion of STRO-1 positive cells in tooth-derived stem cell cultures during the osteogenic differentiation process.

There are also hardly any data available regarding expression of nestin and vimentin during the osteogenic differentiation of DSCs *(Morsczeck, Moehl et al, 2005).*

1.2.3. Expression of osteogenic marker genes during differentiation

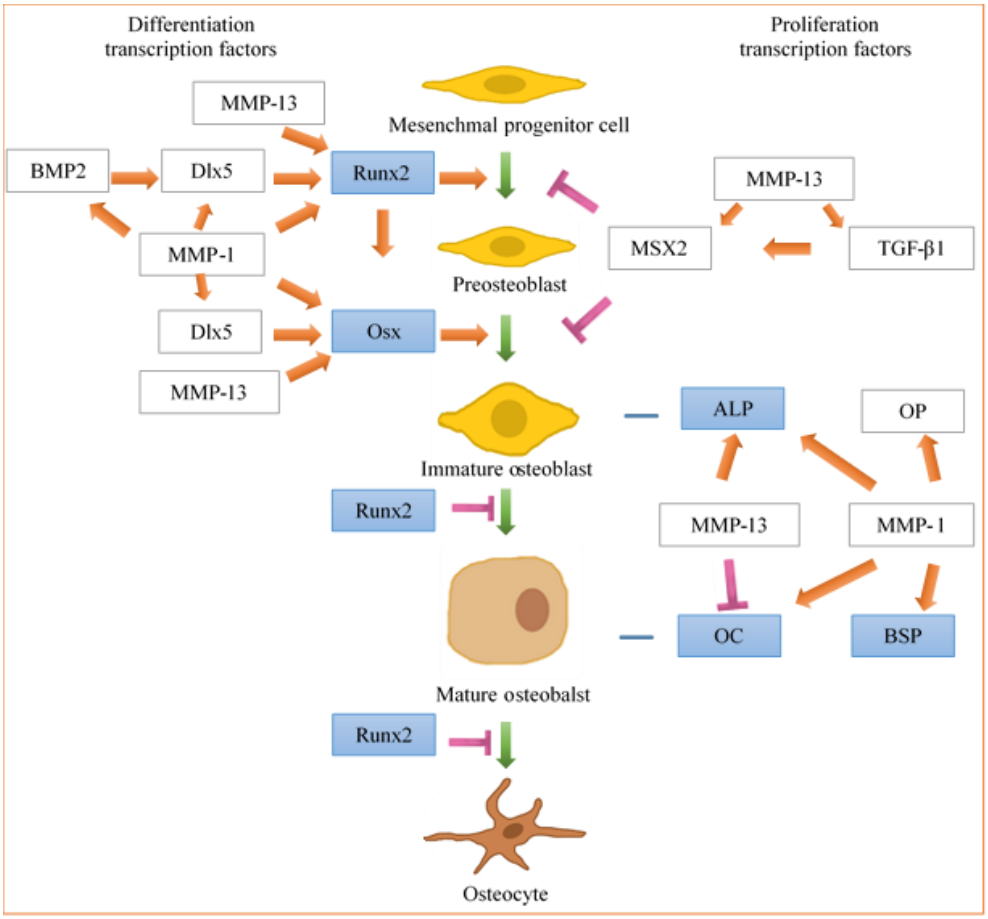

Figure 2

Schematic depiction of the regulation of bone differentiation in mesenchymal progenitor cells, in which the proteins we examined at the mRNA level by qPCR are marked in blue. (*Illustration by the author, based on the figures*: *Komori et al, 2006, Ryoo et al, 2006, Hayami et al, 2011)*

According to the literature, changes in the mRNA levels of some osteogenic marker genes in relation to osteogenic differentiation have been investigated under different conditions and different time points, making it difficult to compare these studies. The aim of our research group was to determine the maturation status of the three dental stem cell types by qPCR studies, based on time-dependent changes in the expression of Runx2 (Runt-related transcription factor 2), SP7 (osterix), ALPL (alkaline phospatase), BGLAP (bone gamma-carboxyglutamic acid-containing protein−osteocalcin), and IBSP (bone sialoproteine) genes simultaneously. Figure 2 is a schematic illustration of the regulation of osteocyte differentiation, highlighting in blue the proteins whose mRNA level changes were studied in our experiments with primary DFSC, PDLSC, and DPSC cultures.

The Runx2 gene is located in human chromosome 6; it encodes the Runx 2 protein and is considered a key transcription factor for osteoblast differentiation. However, Felthaus et al. demonstrated that DFSCs can be differentiated into the osteogenic direction independently of Runx2 *(Felthaus et al, 2014).*

The SP7 gene is located in human chromosome 12; it encodes the Osterix (Osx) protein and also plays an important role in bone formation, as demonstrated by Nakashima et al. in SP7 knockout mice *(Nakashima et al, 2002).*

The ALPL gene is located in human chromosome 1; it encodes the alkaline phosphatase protein (ALP), which is responsible for the mineralization ability of DSCs.

The BGLAP gene can also be found in human chromosome 1, and it encodes the osteocalcin (OC) protein. It was first identified in chicken bones as a calcium-binding protein *(Hauschka and Reid 1978)* but is found in both bones and dentin.

We also examined IBSP in our study, which is located in human chromosome 4 and encodes bone sialoprotein (BSP), which constitutes about 8% of all non-collagen proteins in the bone and cement *(Fisher et al, 1990).*

Therefore, the main purpose of our study was to follow the expressional changes of these genes by monitoring mRNS levels in dental stem cell cultures from three different sources: dental follicle stem cells, periodontal ligament stem cells, and dental pulp stem cells. We also aimed to compare the osteogenic process of these three stem cell types in many other respects (including ALP activity and mRNA expression of osteogenic marker genes) under standardized circumstances.

1.3. Oral antiseptics in dental practice

Disinfectants are regularly used in conventional dental care, for example during endodontic treatments, during periodontal and oral hygiene treatments, and during cariological treatments. Biofilms on different surfaces of the oral cavity are composed of several pathogens that require a broad spectrum of antibacterial antiseptics to be eliminated. When selecting antiseptics, it is important that oral pathogens should have little or no resistance to them. Examples that fulfil these requirements are hydrogen peroxide (H_2O_2) and chlorhexidine (CHX), which are very commonly used in dentistry. However, we must not forget that antiseptics are toxic not only to bacteria but also to the host, so their therapeutic use is limited. Thus, scientific research is looking for further possibilities for even more ideal disinfectants. Recently, the known biocide chlorine dioxide (ClO_2), has come to the foreground. It was first proposed in dentistry to disinfect the air in dental offices *(Kuroyama et al, 2010)* and the surfaces of dental instruments. Several studies have demonstrated the antimicrobial effects of ClO_2 against oral pathogenic bacteria *(Simpson et al, 1993, Herczegh, Ghidan et al, 2013, Herczegh, Gyurkovics et al, 2013).*

1.3.1. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is a pale blue liquid or colorless in a dilute solution. It is one of the oldest disinfectants in dentistry, and is used in various concentrations. Today, a 3% solution is the most commonly used; the maximum concentration allowed in the EU is 6% (Cosmetics Regulation 2012/84/EU), but a 38% solution can be used for teeth whitening by dentists in the USA *(Kinomoto et al, 2001).* Patient-derived periodontal ligament cells were studied by Kinomoto Y. et al. in comparing the toxic effects of H_2O_2 and sodium perborate. H_2O_2 is highly toxic to periodontal ligament cells even in very low concentration (0.03 %) after 24-hour exposure *(Kinomoto et al, 2001).* It has been demonstrated that the viability of human periodontal ligament cells is reduced by H_2O_2 due to an increase in RANK ligand expression *(Pi et al, 2007).*

1.3.2. Chlorhexidine (CHX)

Chlorhexidine is a biguanide-type antiseptic and antimicrobial agent that is effective against Gram-positive and Gram-negative, facultative anaerobic and aerobic bacteria, and yeasts. It has been used as a gold standard in dentistry for more than twenty years due to its bactericid and bacteriostatic effect; it also has an antiplaque effect *(Jones et al, 1997).* Presently, in dental practice a 0.05-0.2% solution of CHX as a mouthwash and a 0.2-6% solution as a root canal flush are commonly used. According to experimental data, the proliferative activity of human gingival fibroblast cells depends on the concentration of chlorhexidine but is independent of exposure time. It has also been demonstrated that CHX reduces the production of collagen and non-collagen proteins in fibroblast cells even at low concentrations (0.0001%) *(Mariotti and Rumpf 1999).* Experiments on equine fibroblasts have shown that the survival rate of these cells is in inverse proportion to the CHX concentration *(Redding and Booth 1991).* Other cytotoxicity studies on PDL cells have found that the effect of CHX is associated with inhibition of protein synthesis and that it is time- and dose-dependent *(Chang et al, 2001).* Significantly fewer gingival fibroblasts adhered to the root surface of a 0.12% CHX-treated tooth in one hour than to the untreated ones *(Alleyn et al, 1991).* Other research groups did not experience a significant change in gingival fibroblast and periodontal ligament cell adhesion or proliferation in the 0.12% CHX-treated root canal surface model, whereas when applying 0.2% CHX the morphology of the cells changed significantly *(Cline and Layman 1992).*

1.3.3. Chlorine dioxide (ClO_2)

Chlorine dioxide is a yellowish-green, oxidative gas, which has been known since the early 1800s. As early as 1983, chlorine dioxide was described as an “ideal biocidal” compound *(Simpson et al, 1993).* Eleven disinfectants were tested by Tanner et al. using Staphylococcus aureus, Pseudomonas aeruginosa, and Saccharomyces cerevisiae, where the greatest biocidal effect was achieved by the chlorine dioxide-containing disinfectant *(Tanner et al, 1989).* Others in a polymicrobial biofilm tooth model system compared

the effects of ClO_2 with CHX (among others), and both disinfectants showed a similarly good bactericidal effect *(Lundstrom et al, 2010).*

Several research groups have investigated how water-soluble ClO_2 exerts its biocidal and antimicrobial effects. Tyrosine, tryptophan, and cysteine molecules, which are oxidized by ClO_2, play a key role in the mechanism of action *(Napolitano et al, 2005, Ison et al, 2006, Napolitano et al, 2006, Stewart et al, 2008).* Since ClO_2 is a strong oxidant, it denatures proteins and thus exerts its antimicrobial effect *(Ogata 2007).* In 2012, it was shown that ClO_2 oxidizes a tryptophan residue in spike proteins of the inflenza virus *(Ogata 2012),* thereby killing the virus. Interestingly, the coronavirus SARS-CoV-2 spike protein also contains cysteine, tyrosine, and tryptophan residues (*Tao et al, 2020*), so this spike protein supposedly can also be degraded by ClO_2 *(Kaly-Kullai et al, 2020).*

But the use of ClO_2 as a disinfectant was hampered by the fact that it could not be produced in a stable state until 2006. At this time, a new membrane separation method was developed by Prof. Zoltán Noszticzius *(Noszticzius et al, 2008)* at the Technical University of Budapest. Applying this technique, a much more stable, hyperpure solution of ClO_2 can be produced *(Koncz et al, 2017).* In Hungary, the hyperpure chlorine dioxide solution prepared by this method is available under the brand name Solumium, which has been marketed and used in dental practice since 2008 *(Csikány et al, 2009).* A research team at Semmelweis University has detailed the antimicrobial efficacy of hyperpure ClO_2 against oral pathogenic bacteria in two scientific articles *(Herczegh, Ghidan et al, 2013, Herczegh, Gyurkovics et al, 2013).* Other groups have shown that chlorine dioxide is effective not only against bacteria but also against bacteriophages *(Grunert et al, 2018).*

A special feature of ClO_2 is that it is a size-selective antimicrobial agent, and bacteria are unable to develop resistance against it *(Noszticzius et al, 2013).* Several factors are involved in size selectivity. First, viruses are the smallest in size; their outer protein coat is destroyed by ClO_2 *(Kaly-Kullai et al, 2020).* As ClO_2 is very volatile, it is effective for only a few minutes. This time period is much longer than the time required to kill bacteria, however it is not long enough for ClO_2 to penetrate more deeply into tissue – it is therefore not harmful to humans. Glutathione in bacteria and human cells can bind the active ClO_2 at high rates *(Ison et al, 2006).* The next

explanation for size selectivity is that while the supply of glutathione by bacteria is soon depleted, the amount of glutathione in human cells is much higher *(Kaly-Kullai et al, 2020)*. In addition, other antioxidants are also present in human cells and tissues *(Forman et al, 2009)*, so they inactivate ClO_2 more intensively. Interestingly, volunteers drank the ClO_2 aqueous solution daily for 12 weeks, and found no negative side effects *(Lubbers et al, 1982)*.

Based on the properties mentioned so far, hyperpure chlorine dioxide solutions might be the most ideal disinfectants for both medical and dental use. But one of the biggest obstacles to clinical application in dentistry is the lack of data on the possible toxicity of hyperpure ClO_2 on cells in the human oral cavity.

It was an honour for me to participate in the cytotoxic study of this Hungarian invention, Solumium. From the three dental mesenchymal stem cells I examined, PDLSCs were selected as a test cell type. On one hand, this was due to the location of these stem cells in the oral cavity. In contrast, DFSCs are surrounded by alveolar bone, while DPSCs are surrounded by hard tissues of the tooth (dentin and enamel). On the other hand, PDLSCs are actually exposed to disinfectants when these are used to rinse the periodontal sulcus during periodontal treatment. During my work I compared hyperpure chlorine dioxide with two disinfectants used in dental practice for decades – hydrogen peroxide and chlorhexidine – investigating their effects on human PDLSCs. I examined the viability and morphology of PDLSCs, as well as their protein marker expression by immunocytochemistry.

2. OBJECTIVES

During my doctoral work, my aims were the following:

0. Isolating and cultivating of ***PDLSC*** and ***DPSC*** primary dental stem cell cultures; establishing, isolating, and cultivating protocols for ***DFSC*** primary cell cultures.

Comparing ***DFSC***, ***PDLSC***, and ***DPSC*** primary cell cultures during 21-day osteogenic differentiation at several different time points:

1. assessing changes in ***cell number*** and in ***alkaline phosphatase enzyme activity***,

2. examining ***mineralisation***,

3. determining ***protein-level*** changes of markers characteristic of MSC,

4. examining the ***expression of genes***, regulating different stages during osteogenic differentiation.

5. Investigating the effects of H_2O_2, ***CHX*** and hyperpure ClO_2 (active components in disinfectant mouthwashes) on cell morphology, viability, and the presence of stem cell markers in ***PDLSC*** primary cell cultures.

3. METHODS

Here is a brief summary of all the methods. Both from my first-authored and co-authored articles, as well as the methodological parts that are not included in the articles.

For ***cell isolation*** of DPSCs, PDLSCs and DFSCs from impacted wisdom teeth surgically extracted at the SE Department of Oral Diagnostics, a protocol already used in SE Department of Oral Biology was applied. ***Cultivation*** was performed in αMEM-based culture medium containing 10% FBS. For all ***morphological studies***, I used an inverted phase contrast microscope, a CCD camera and the Scion image processing software *(Perczel-Kovach et al, 2021;Lang et al, 2021).*

For the three week-long-***osteogenic differentiation***, DFSCs, PDLSCs and DPSCs from the third passage were seeded at a ratio of 104 cells/cm2 . 24 hours after seeding, the expanding medium was replaced with αMEM-based control and osteogenic medium containing 1% FBS. Osteogenic differentiation was induced by a combination of ascorbic acid 2-phosphate, β-glycerophosphate and dexamethasone. Measurements were taken at the start of differentiation (day 0) and on days 7, 14 and 21 *(Perczel-Kovach et al, 2021).*

DNA quantification measurements were performed using 96 well plates seeded with DFSCs, PDLSCs and DPSCs. DNA was isolated from these cells using Nucleospin Tissue kit according to the manufacturer's protocol. DNA concentration in the cell culture is proportional to the cell number *(Perczel-Kovach et al, 2021).*

A colorimetric method was applied to measure the ***ALP enzyme activity*** using 96 well plates seeded with DFSCs, PDLSCs and DPSCs. The obtained ALP activity values were normalized to DNA content and expressed as mM pNP/h/μg DNA *(Perczel-Kovach et al, 2021).*

When ***measuring Ca^{2}+ concentration***, the inorganic calcium salts deposited around the DFSCs, PDLSCs and DPSCs were dissolved in 0.5 M HCl. The Ca concentration was determined in each well using an Abcam calcium detection kit and the supplied standard solution with an Ultrospec III spectrophotometer *(Perczel-Kovach et al, 2021).*

During ***von Kossa staining***, after fixation with 70% ethanol, the differentiated DFSCs, PDLSCs and DPSCs were treated with 5% silver nitrate under bright light.

Then 5% sodium thiosulphate was used to fix the color change *(Perczel-Kovach et al, 2021).*

Changes in mRNA levels were measured on days 0, 7 and 14 of the osteogenic groups using our previously published qPCR protocol. Measurements were performed using a StepOne® ***Real Time PCR*** system. The following genes were tested: Runx2, bone sialoprotein, alkaline phosphatase, osterix, osteocalcin and the endogenous control glyceraldehyde-3-phosphate (GAPDH) *(Perczel-Kovach et al, 2021).*

The stem cells were plated on 8-chamber slides, fixed with 4% PFA at the time of investigations and stored at 4°C until simultaneous ***immunocytochemical staining***. The primary antibodies were against: CD105, CD90, vimentin, nestin, STRO-1, ON and BSP. Secondary antibodies were Alexa Fluor 488- conjugated goat anti-rabbit IgG, anti-mouse IgG and IgM. The plates were coated with ProLong Gold antifade reagent with DAPI. During osteogenic differentiation, STRO-1 + cells were quantified *(Perczel-Kovach et al, 2021; Lang et al, 2021).*

The effects of H_2O_2 and CHX on PDLSCs were compared with those of hyperpure ClO_2 ***in the cytotoxity study***, mimicking dental treatment with a 10-minute-long exposure time. On one hand, we 7 investigated concentration-dependent toxicity, on the other hand, we compared the effects of concentrations used in dental practice (0.3% H_2O_2, 0.02% CHX, 0.0025% and 0.00025% hyperpure ClO_2) on PDL stem cells from two different passage numbers (P3, P7) *(Lang et al, 2021).*

In our department, we have routinely used the WST-1 ***cell viability assay*** to test cell viability of PDLSCs. The assay is based on the measurement of mitochondrial dehydrogenase enzyme activity. The changes of absorbance contributing to color reaction was measured using a microplate reader *(Lang et al, 2021).*

Data were expressed as arithmetic mean ± standard errors of the mean (SEM) and statistical analysis was performed using Kruskal Wallis non-parametric ANOVA, where differences with $p<0.05$ were considered significant *(Perczel-Kovach et al, 2021; Lang et el, 2021).*

4. RESULTS

4.1. Results of long-term cultivation of the three dental stem cell cultures

4.1.1. Morphological changes during long-term cultivation

After isolation, dental mesenchymal stem cells adhered within 8 hours and distributed throughout the surface of the culture vessels. During the following days, the fibroblast-like DSCs first formed small colonies and then interconnected, forming chain-line structures by the fourth or fifth days (Figure 3 row A). Later, 9 to 11 days after isolation, each cell culture usually reached a 70-80% confluency level (Figure 3 row B).

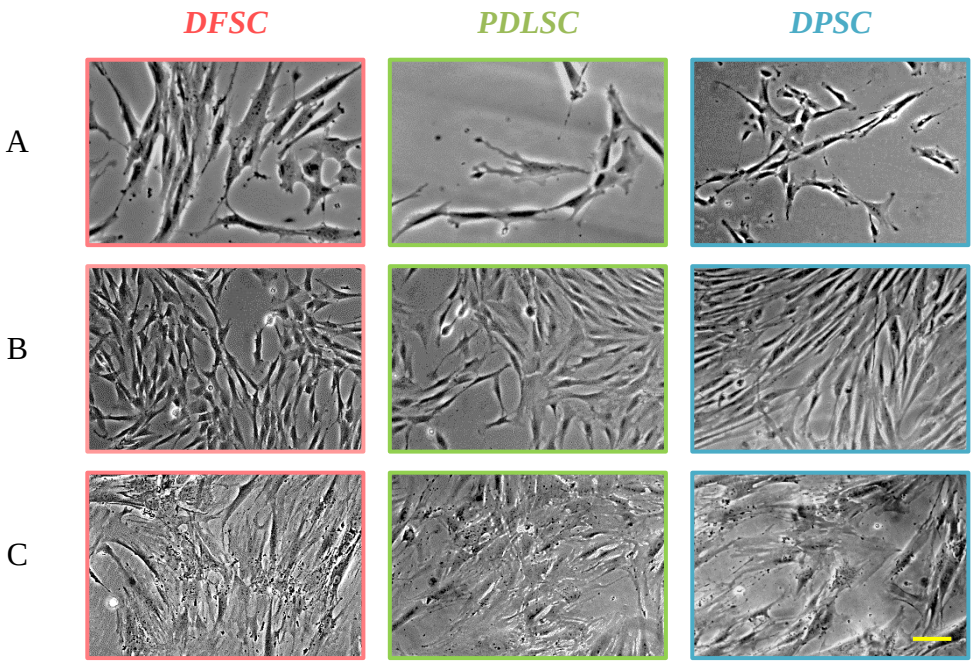

Figure 3

Phase contrast microscopic images during culturing of DFSCs, PDLSCs, and DPSCs. Four days after isolation (row A), before passage at 70-80% confluency (row B) and before passage 17 (row C) when all three primary cell cultures had already lost their fibroblast-like morphology. All the photomicrographs were taken at the same magnification. The bar indicates 100 µm.

All three primary cell cultures retained their fibroblast-like morphology until higher passage numbers (around passage 10), and then gradually lost this morphology. Flattened, spreading cells appeared in passages P9-12, and the ratio of these senescent cells increased remarkably during further passages until almost all cells showed such a morphology (passage 17 – Figure 3 row C).

4.1.2. Immunocytochemical results of undifferentiated cells

Comparing DFSC, PDLSC, and DPSC cells in passage P3 by immunocytochemistry, the following results were found. Among the mesenchymal stem cell markers, STRO-1 was expressed by only less than one-third of the cells in all three primary cell cultures. After quantification, we detected the following ratios of STRO-1 positive cells: DFSC – about 10%, PDLSC – about 15%, DPSC – about 20%. Positive staining was observed in the majority of cells for the mesenchymal marker vimentin. Comparing primary cell cultures with each other (Figure 4), we found only a subtle difference in the intensity of staining (DPSC > DFSC > PDLSC).

For the ectodermal stem cell marker (nestin), positive staining was obtained for practically all cells of DFSC, PDLSC, and DPSC cultures, the latter intensity being slightly greater (Figure 4). Positive staining similar to nestin was also observed for the CD90 protein markers (Figure 4).

Among the osteogenic markers, the presence of ON and BSP was examined. Both markers showed positive staining in the majority of the cells. Comparing the primary cell cultures, only a modest difference in staining intensity was observed (Figure 4).

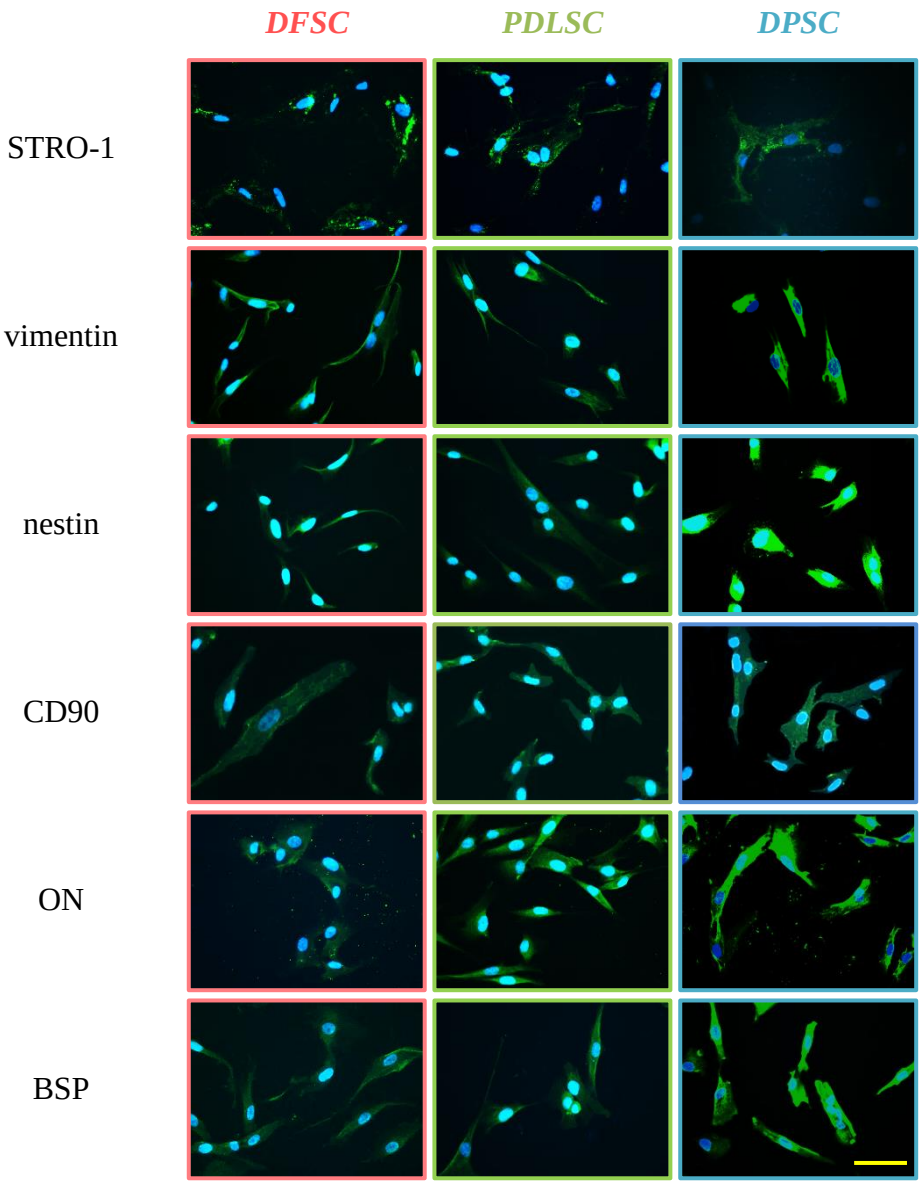

Figure 4

Immunofluorescent detection of markers: STRO-1, vimentin, nestin, CD90, osteonectin (ON) and bone sialoprotein (BSP) in DFSC, PDLSC, and DPSC cultures (passage 3). All the photomicrographs were taken at the same magnification. The bar indicates 50 μm. Green indicates the specific immunostaining (Alexa Fluor 488) while blue indicates cell nuclei (DAPI).

4.2. Study of dental follicle stem cells, periodontal ligament stem cells, and dental pulp stem cells during three-week osteogenic differentiation

4.2.1. Changes in cell number during osteogenic differentiation

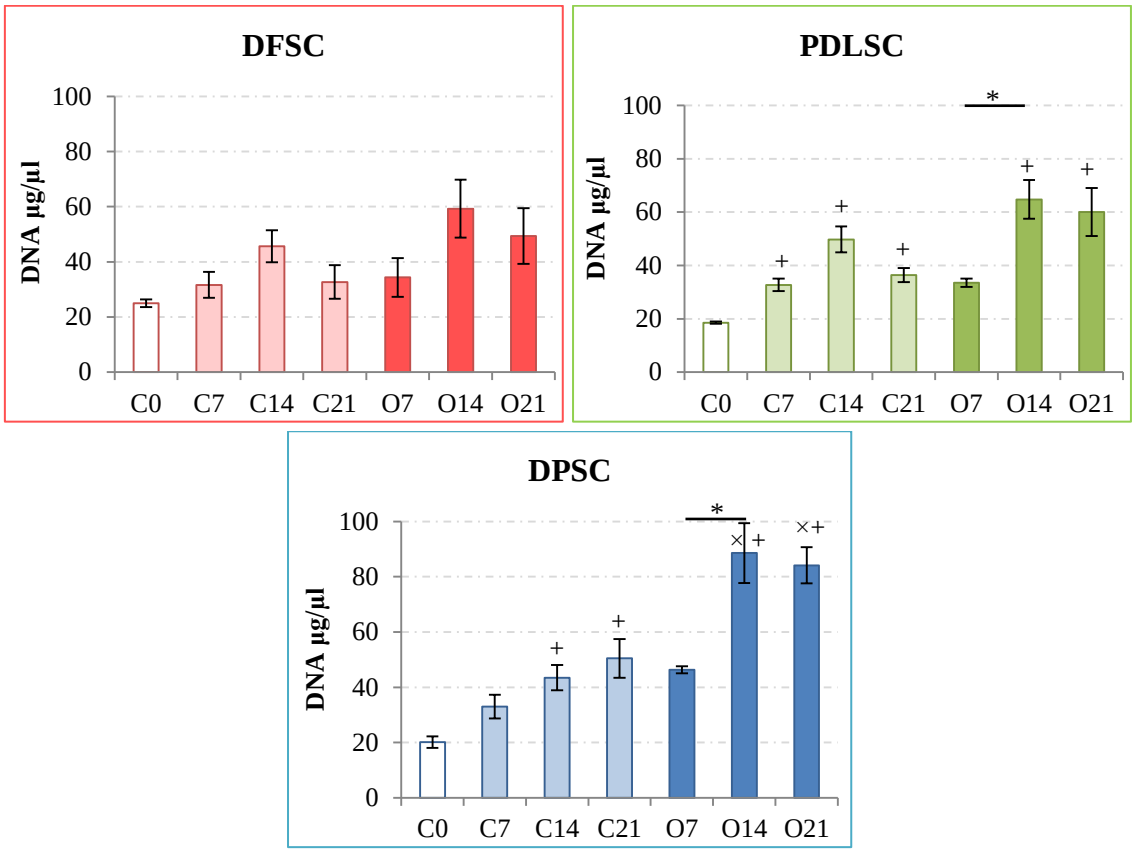

Figure 5

DNA content as an index of cell number during 21-day osteogenic differentiation in the control (C) and in the osteogenic (O) medium, on days 0, 7, 14, and 21. The primary cell cultures tested were: dental follicle stem cell (DFSC), periodontal ligament stem cell (PDLSC), and dental pulp stem cell (DPSC). The data are given as an arithmetic mean ± SEM (standard error of the mean) $p<0.05$ ANOVA (Kruskal-Wallis) * within group $^{+}$ to day 0 $^{\times}$ to the same day *(Perczel-Kovach et al, 2021).*

Changes in cell number were determined during the three-week osteogenic differentiation period by measuring DNA concentration. The change in DNA concentration of the three primary mesenchymal stem cell cultures is shown on Figure 5.

In the PDLSCs we observed a significant continuous increase until day 14 in both the osteogenic and control groups, and then a small decrease by day 21, when values were still significantly higher compared to day 0 (C0). The same trends were observed for DFSC (Figure 5).

In the case of DPSCs, we detected a continuous increase in cell number in the control group, which was statistically significant on days 14 and 21 compared to C0. In the osteogenic group, a more intense significant increase was observed by day 14, and then this elevation had stopped by day 21.

4.2.2. Study of mineralization during osteogenic differentiation

Two methods were used to detect mineralization. Neither von Kossa staining nor calcium concentration measurement showed any sign of mineralized matrix formation in the control groups. However, in the osteogenic groups, calcium deposits were detectable by day 14 in all three primary cell cultures.

Calcium deposits stained with von Kossa staining can be seen in Figure 6A as dark brown spots. The difference between DFSC and DPSC cultures was already visible to the naked eye: the DFSC cultures showed much more intense staining.

By measuring calcium concentrations on day 14, approximately 0.03 μg Ca/μg DNA was found for all three mesenchymal primary stem cell cultures. On day 21, values of a higher order of magnitude were measured: about 0.3-0.7 μg Ca/μg DNA. The calcium concentration of the DFSC cultures was significantly higher compared to those of DPSCs. (Figure 6B)

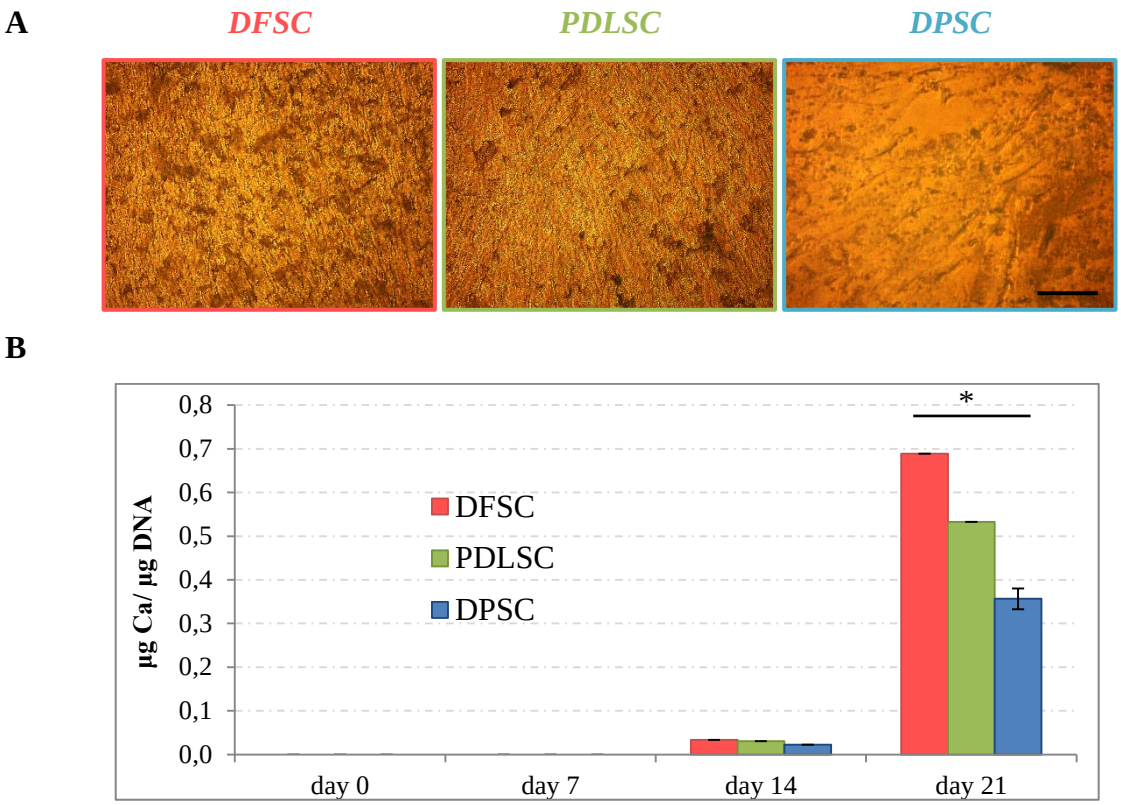

Figure 6

Results of the osteogenic groups: von Kossa staining on day 21 (**A**) and calcium concentration measurements (**B**). Dental follicle stem cells (DFSC), periodontal ligament stem cells (PDLSC), and dental pulp stem cells (DPSC) were compared. All the photomicrographs were taken at the same magnification. The bar indicates 100 µm. The data are given as arithmetic mean ± SEM (standard error of the mean) * $p<0.05$ ANOVA (Kruskal-Wallis) *(Perczel-Kovach et al, 2021).*

4.2.3. Immunocytochemical results during osteogenic differentiation

The following results were obtained during immunocytochemical studies. All of the mesenchymal stem cell markers (vimentin and nestin), and osteogenic protein markers (ON, BSP) gave positive staining and homogenous distribution throughout the cell cultures in both the control and osteogenic groups (Figure 7A).

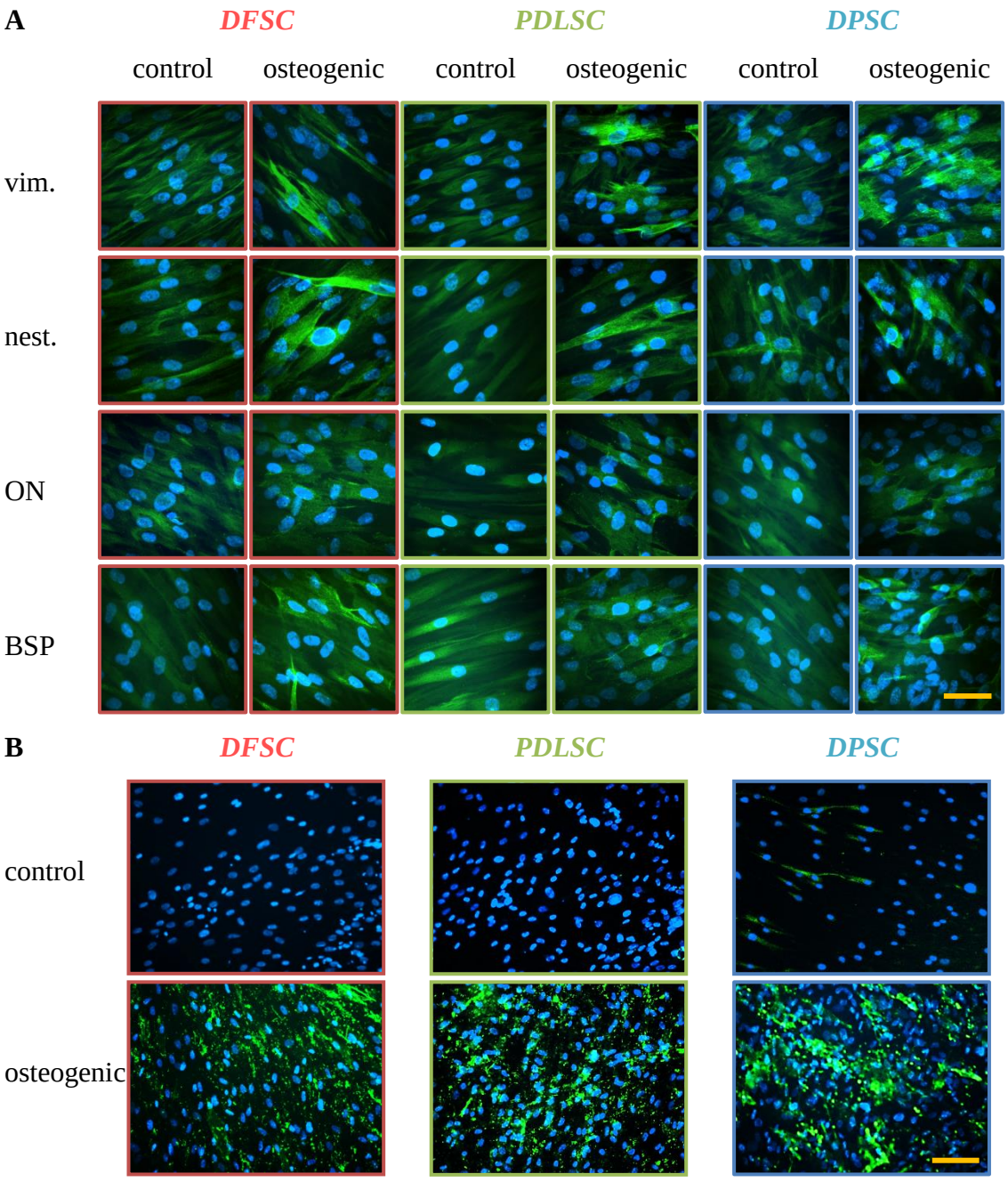

Figure 7

Comparison of control and osteogenic groups by immunofluorescent staining of markers vimentin, nestin, ON, BSP (**A**), and STRO-1 (**B**) on day 21. Dental follicle stem cell (DFSC), periodontal ligament stem cell (PDLSC), and dental pulp stem cell (DPSC) cultures were compared. All the photomicrographs were taken at the same magnification within images "A" and "B", respectively. The bars indicate: (A) 100 µm, (B) 200 µm. Green indicates the specific immunostaining (Alexa Fluor 488), and blue indicates cell nuclei (DAPI) *(Perczel-Kovach et al, 2021).*

According to Figure 7A, we can observe that at day 21 there was no remarkable difference in staining intensity between either the control or osteogenic groups of the same cell type or between the three tooth-derived progenitor types.

In contrast, we found significant differences in the expression of the STRO-1 marker (Figure 7B), therefore, these differences were also quantified (Figure 8).

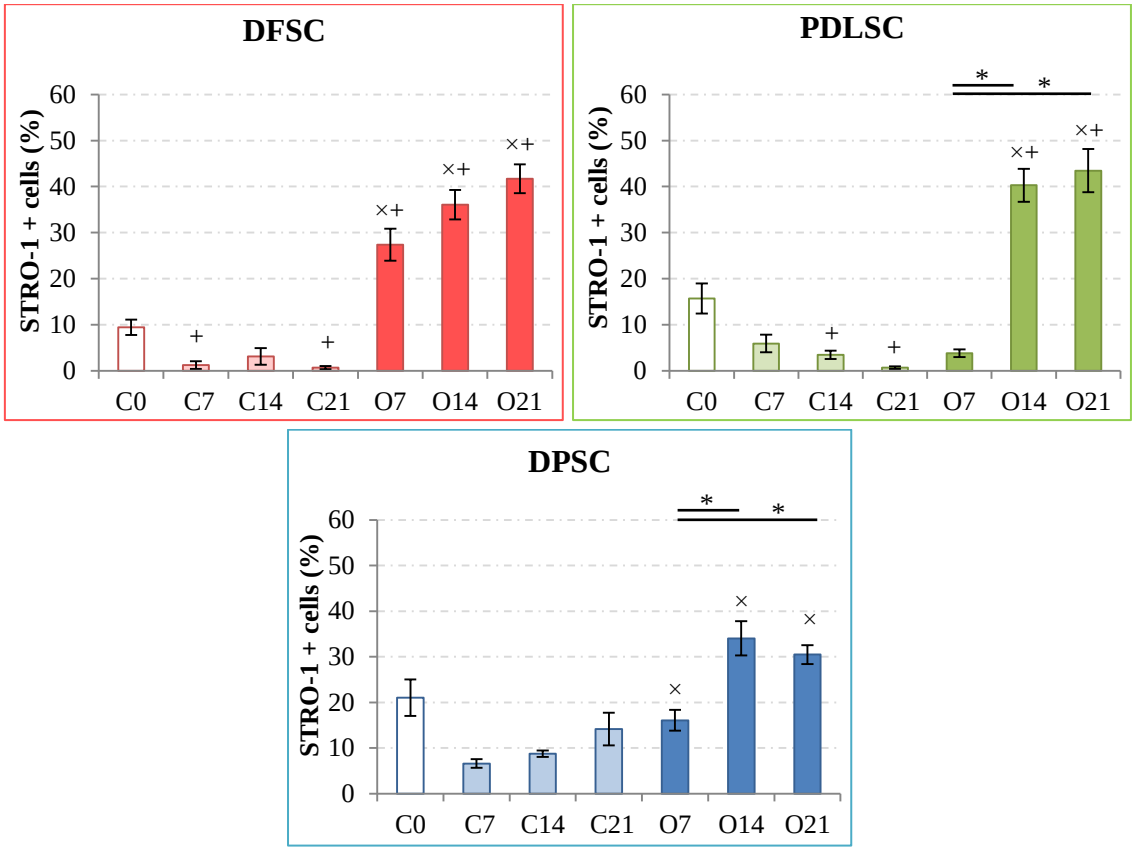

Figure 8

Result of quantification of the proportion of STRO-1 positive cells in immunocytochemically stained dental follicle stem cell (DFSC), periodontal ligament stem cell (PDLSC), and dental pulp stem cell (DPSC) cultures. The data are given as arithmetic mean ± SEM. $*^{\times+}p<0.,05$ ANOVA (Kruskal-Wallis); *within group; $^{+}$to day 0; $^{\times}$to the same day *(Perczel-Kovach et al, 2021).*

At the beginning of the differentiation (C0), the proportion of STRO-1 positive cells was around 10, 15, and 20% for DFSC, PDLSC, and DPSC, respectively. In the

DFSC and PDLSC cultures, the proportion of positive cells was significantly reduced in the control group. For DPSCs, only a decreasing trend was observed. In the osteogenic groups, the proportion of STRO-1-positive cells increased by day 21 significantly in all three primary cell cultures, to 40, 45, and 30% for DFSC, PDLSC, and DPSC, respectively.

4.2.4. Changes in ALP activity during osteogenic differentiation

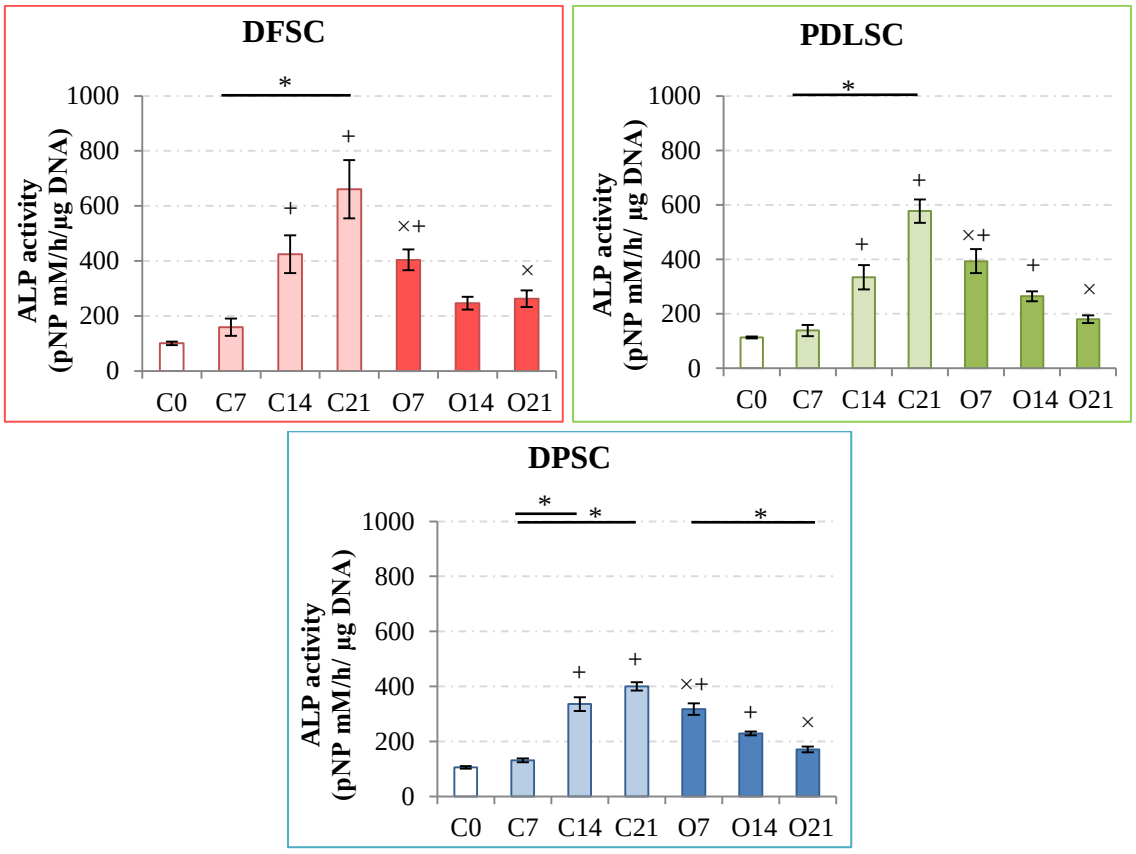

Figure 9

Alkaline phosphatase activity changes during the 21-day osteogenic differentiation in the control (C) and osteogenic (O) medium on days 0, 7, 14, and 21. The tested celltypes were: dental follicle stem cell (DFSC), periodontal ligament stem cell (PDLSC) and dental pulp stem cell (DPSC). Mean ± SEM; $*^{\times+}p<0.05$ ANOVA (Kruskal-Wallis); *within group; $^{+}$to day 0; $^{\times}$to the same day *(Perczel-Kovach et al, 2021).*

The ALP activity values of the dental mesenchymal stem cells were monitored by measuring DNA content (Figure 9). On day zero, relatively low ALP activity was measured for all three primary cell cultures. In the control groups, a continuous increase was measured (significant difference between C0 and day 14) in contrast to the osteogenic group, where the marked (significant) increase between day 0 and 7 was followed by a decrease. Comparing the three primary cell cultures with each other, we can set up the series DFSC > PDLSC > DPSC in terms of the extent of the changes.

4.2.5. Results of the quantitative real-time PCR analysis

RT-qPCR analysis was performed in all three primary cell cultures on days 0, 7, and 14 of the osteogenic group, and the relative quantification (RQ) values were normalized to the values determined on day zero (Figure 10).

The mRNA levels of the Runx2 gene increased three- to four-fold by day 7 in all three primary cell cultures, and then decreased by day 14. In the case of osterix (Osx), an approximately twofold increase was measured in DFSCs and PDLSCs by day 7, then the data fell below baseline by day 14. A gradual decrease was measured for DPSCs during the first two weeks of differentiation. For BSP, we observed a remarkable increase (eightfold) by day 7 only in the case of DPSCs, and this was followed by a decrease. In PDLSCs, only a slight increase (around twofold) of Runx2 mRNA level was measured, while a gradual decrease was found for DFSCs (Figure 10).

Examining osteocalcin (OC) mRNA expression, we observed only minimal changes in DFSC and PDLSC cultures. However, much higher expression levels were detected in DPSCs. By day 7 a rapid 15-fold increase, and by day 14 a more than 130-fold increase were measured compared to day zero. Similar levels were obtained for DFSCs and PDLSCs when examining the ALP gene. By day 7, there was an about 120- to 130-fold increase followed by a decrease to about half this value by day 14. The ALP mRNA level of DPSCs increased approximately 1200-fold by day 7 and did not decrease but rather increased slightly further by day 14 (Figure 10).

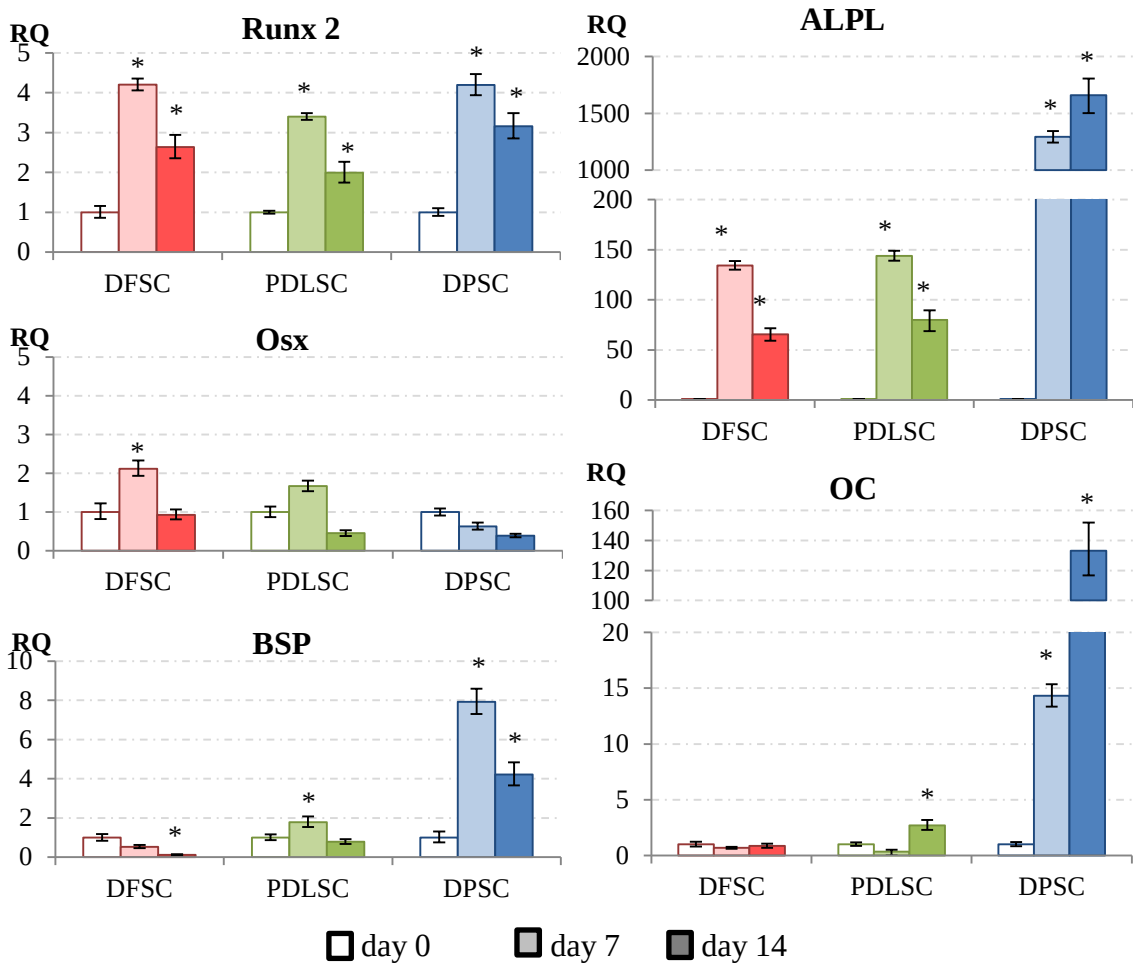

Figure 10

Results of RT-qPCR analysis of mRNA expression of Runx2, osterix (Osx), bone sialoprotein (BSP), alkaline phosphatase (ALPL), and osteocalcin (OC) on days 0, 7, and 14 of osteogenic differentiation in cultures of dental follicle stem cell (DFSC), periodontal ligament stem cell (PDLSC), and dental pulp stem cell (DPSC). Data are normalized to GAPDH mRNA expression. *p<0.05 to day 0 *(Perczel-Kovach et al, 2021).*

4.3. Effects of chlorhexidine, hydrogen peroxide, and hyperpure chlorine dioxide on periodontal ligament stem cells

4.3.1. Cell morphology during toxicity studies

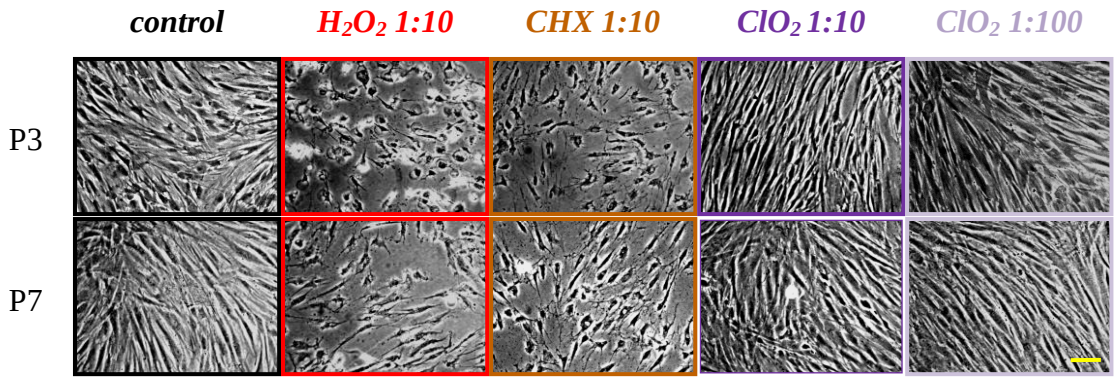

Figure 11

Phase contrast microscopic investigation of PDLSC cultures from passage 3 (P3) and passage 7 (P7). Morphology of the untreated control and 48 hours after the 10-minute treatments with 0.3% H_2O_2 (1:10), 0.02% CHX (1:10), 0.0025% ClO_2 (1:10), and 0.00025% ClO_2 (1:100). All the photomicrographs were taken at the same magnification. The bar indicates 100 µm *(Lang et al, 2021)*.

While studying the effects of mouthwashes (H_2O_2, CHX, ClO_2) on PDLSCs, we observed the following morphological changes. Forty-eight hours after treatment with the concentration applied in the dental praxis (1:10), the untreated cells showed a healthy fibroblast-like morphology in both tested passage numbers (Figure 11, control P3 and P7). PDLSC cultures responded to both disinfectants, 0.3% H_2O_2 (1:10) and 0.02% CHX (1:10), with cell morphological changes and drastic cell depletion. The original spindle-shape was replaced by a rounded morphology and their processes became much thinner (Figure 11, H_2O_2 1:10 and CHX 1:10 column). Taken together, these observations indicated a drastic decrease in cell viability. Cell cultures treated with 0.0025% (1:10) and 0.00025% (1:100) concentrations of ClO_2 showed no differences in cell morphology or cell growth compared to the control (last two columns of Figure 11).

4.3.2. Changes in cell viability during toxicity studies

Investigation of the concentration-dependence of toxicity

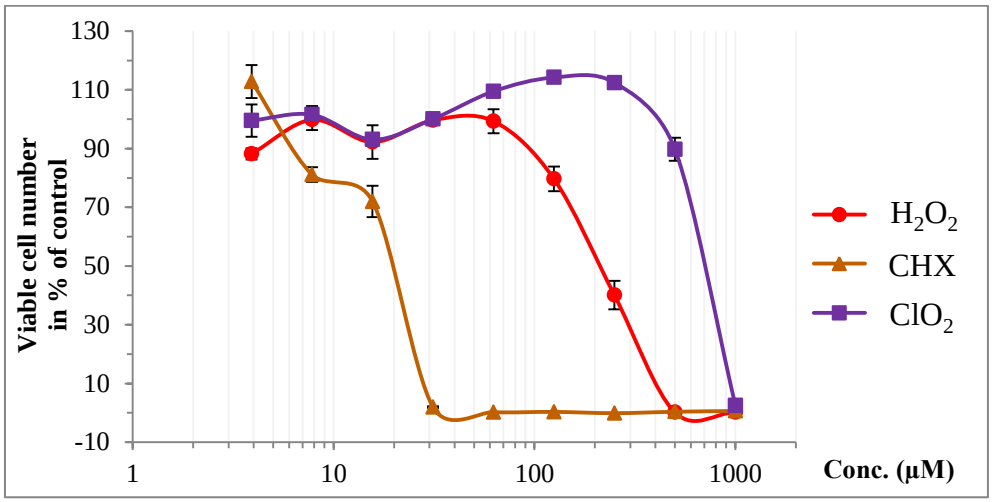

Figure 12

Dose-response curves of PDLSC cultures treated with H_2O_2, CHX, and ClO_2. Viability analysis of cell cultures 48 hours after the 10-minute treatments by WST-1 assay *(Lang et al, 2021).*

Examining the concentration-viability relationship by WST-1 assay, the following results were obtained (Figure 12). CHX was already unequivocally toxic to PDLSCs at very low concentrations: the calculated IC_{50} value (representing a 50% reduction in viability) was 29.5 µM. The concentration of CHX solution applied in periodontology is 0.25%, which corresponds to 3960 µM. In the case of H_2O_2, a higher concentration was required to reduce viability by 50%: the calculated IC_{50} was 209 µM. The 3.5% H_2O_2 solution routinely used in dental practice corresponds to a 88.240 µM solution. For hyperpure ClO_2, the calculated IC_{50} was 638 µM. In periodontology, the applied concentration of ClO_2 is 0.025%, which corresponds to 3700 µM. This is less than six times higher than its IC_{50}, in contrast to CHX and H_2O_2, where the concentration used in dentistry (µM value) is several hundred times higher than the measured IC_{50} value for PDLSCs (CHX – about 240 times; H_2O_2 – about 420 times).

Examination of cell viability in the context of cell age

The following cytotoxicity results of mouthwashes on PDLSCs from different passages were obtained by the WST-1 assay (Figure 13). Due to the drastic cytotoxic effect of 0.3% H_2O_2, there were no viable cells in either passages (P3 and P7). About 75% of PDLSC P3 cells and about 20% of PDLSC P7 cells treated with 0.02% CHX remained viable. The cells treated with ClO_2 did not show a decrease in viability but a slight (non- significant) increase was observed. PDLSC P3 cells treated with 0.00025% ClO_2 (1:10 ClO_2) showed approximately 20% growth compared to the untreated control (Figure 13).

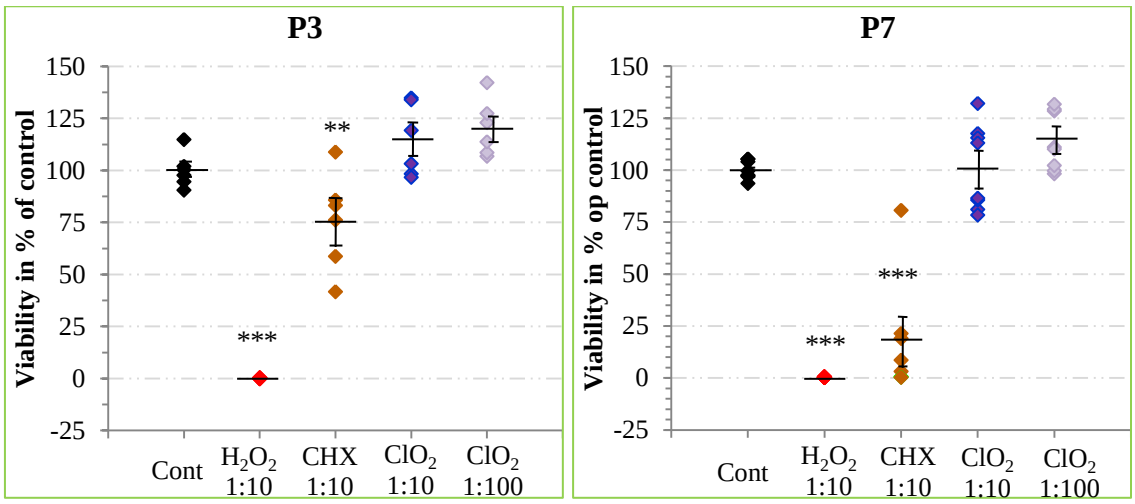

Figure 13

Cell viability of PDLSC cultures from passage 3 (P3) and passage 7 (P7) 48 h after the 10-minute treatment with 0.3% H_2O_2 (1:10), 0.02 % CHX (1:10), and 0.0025% (1:10) or 0.00025% (1:100) ClO_2 The values (±SEM) are expressed in % of the untreated controls. **p<0.01; ***p<0.001 *(Lang et al, 2021)*.

4.3.3. Immunocytochemical results during toxicity studies

In the immunocytochemical studies, the expression of three mesenchymal stem cell markers (STRO-1, CD90, and CD105) was examined as a reference to determine the effect of mouthwashes on PDLSCs at clinically used concentrations on the stemness of

the cell cultures. The expression of these proteins in PDLSC cells was examined at an earlier (P3) and a later (P7) passage.

As the cells were rounded and floated (indicating that they were dead) under the influence of hydrogen peroxide, we could not fix the cells in this group, and thus we could not detect their protein marker expression.

Our indirect immunocytochemistry-based evaluations showed that neither of the other tested mouthwashes (i.e. chlorhexidine and chlorine dioxide) influenced the expression of the above-mentioned stem cell markers in either the P3 or P7 generations of PDLSC cultures. Less than half of the cells were STRO-1 positive, and nearly all of the cells expressed the markers CD90 and CD105 in both the control and treated groups (Figure 14).

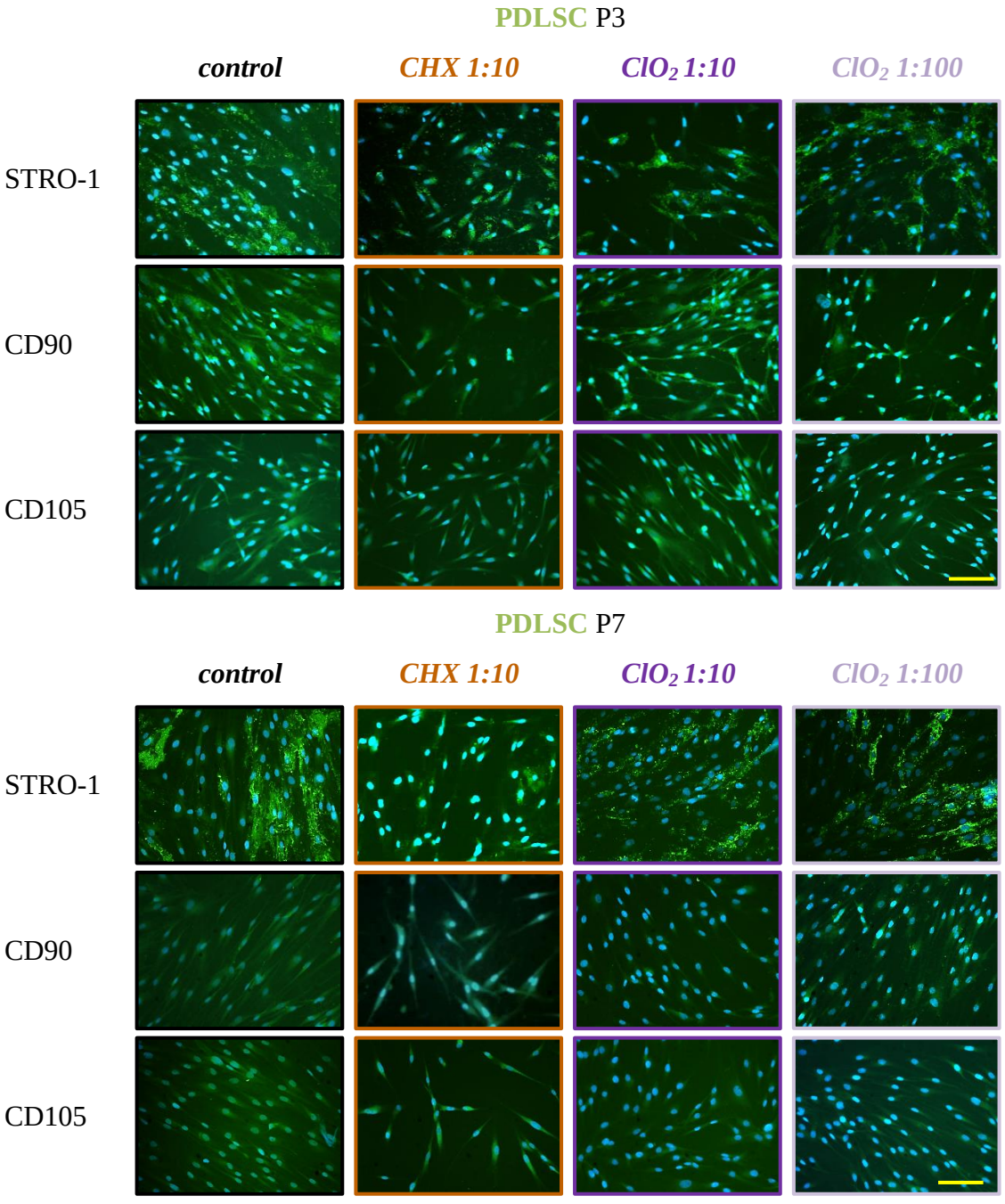

Figure 14

Immunfluorescent staining of mesenchymal stem cell markers STRO-1, CD90, and CD105 in control and treated PDLSC cultures from third passage (P3) and seventh passage (P7) 48 hours after treatment with 0.02% (1:10) CHX and 0.0025% (1.10) or 0.00025% (1:100) ClO_2. All the photomicrographs were taken at the same magnification. The bars indicate 100 μm. Green indicates the specific immunostaining (Alexa Fluor 488) and blue indicates cell nuclei (DAPI) *(Lang et al, 2021)*.

5. DISCUSSION

5.1. Characterization of primary cultures from dental follicle stem cells, periodontal ligament stem cells, and dental pulp stem cells

Like previous members of the research groups working at the Department of Oral Biology *(Kadar et al, 2009, Kiraly et al, 2009)*, we have successfully isolated DPSCs and PDLSCs from human third molars. In addition, I was the first in this department to successfully isolate and culture DFSCs from extracted third molars. These cells are also special to me because I did not just encounter them during isolation, but I was also actively involved in surgical operations in which the impacted third molars were removed at the Section of Dentoalveolar Surgery of Department of Oral Diagnostics.

Examining the three primary stem cell cultures, we found that each of them showed a fibroblast-like morphology until about 10 passages (Figure 3), after which the cells began to show signs of aging. Similar observations have been described by other research groups *(Kawanabe et al, 2010, Dave and Tomar 2018, Wang et al, 2018).*

In accordance with the literature *(Huang et al, 2009, Dave and Tomar 2018, Shuai et al, 2018)*, the cell cultures we examined expressed CD90, vimentin, nestin, ON, and BSP in very high proportion, while STRO-1 was expressed by only a small ratio of cells (Figure 4).

With these experiments, we demonstrated that our isolated cells belonged to dental mesenchymal stem cells.

5.2. Characterization of dental follicle stem cells, periodontal ligament stem cells, and dental pulp stem cells during three-week osteogenic differentiation

In this chapter, I have summarized our study regarding osteogenic differentiation of the three primary dental mesenchymal stem cell cultures (DFSC, PDLSC, and DPSC) that I isolated. The osteogenic differentiation potentials of these three primary stem cell cultures are very similar, but we also found differences between them during the differentiation process at several points *(Perczel-Kovach et al, 2021).*

Several research groups have addressed the osteogenic differentiation of dental mesenchymal stem cells, yet there is no detailed study comparing dental pulp-derived stem cells with other dental primary stem cell cultures while monitoring changes at both the cellular and molecular levels. Winning and colleagues compared cultures from two dental pulps with cultures of periodontal ligament-derived cultures during osteogenic differentiation, but their work lacks an examination of immunomarkers *(Winning et al, 2019)*. There is a research group that presents only qualitative results instead of quantitative changes over time *(Zhang et al, 2018)*. Moreover, the osteogenic differentiation protocols applied on MSCs are highly diverse *(Hoemann et al, 2009)*. Most research teams choose MEM, αMEM, or DMEM as basal media, supplemented with FBS in each case. L-ascorbic acid 2-phosphate, KH_2PO_4, β-glycerophosphate, dexamethasone and 1α-25-dihydroxyvitamin D3 are commonly used supplements for osteoinductive media. Differentiation in the osteogenic direction has been mainly achieved in DPSCs using a very high (20%) FBS concentration *(Laino et al, 2005, d'Aquino et al, 2007, Mangano et al, 2010)*. Regarding the abovementioned supplements, the concomitant use of dexamethasone, β-glycerophosphate, and ascorbic acid is the best characterized *(Langenbach and Handschel 2013)*, which is the reason we also chose this combination. According to the previous findings of our research group, both PDLSC and DPSC can be differentiated in the osteogenic direction at 1% FBS concentration *(Kadar et al, 2009)* while applying the protocol that was used by Kémoun et al. on DFSCs *(Kemoun et al, 2007)*.

The results of DNA quantification which are proportional to cell number (Figure 5) also support our conclusion that a low (1%) FBS concentration does not inhibit cell proliferation, in concordance with a previous study on DPSCs *(Galler et al, 2008)*.

5.2.1. Changes in DNA content, and ALP activity after osteogenic induction

In summary, DNA quantification and ALP activity measurement led to the following conclusions.

The cell number in DFSC, PDLSC, and DPSC cultures showed continuous growth in both the osteogenic and control groups over three weeks, even in media containing

1% FBS, without passaging (Figure 5). Therefore, higher cell density does not inhibit proliferation of these cell types. According to the literature, the concentration of FBS in the osteogenic differentiation medium varies between 1% and 20% *(Gronthos et al, 2000, Nagatomo et al, 2006, Kemoun et al, 2007, Galler et al, 2008, Lin et al, 2008, Wei et al, 2008, Kawanabe et al, 2010, Kraft et al, 2010, Mangano et al, 2010, Yan et al, 2014, Rezai-Rad et al, 2015, Goto et al, 2016)*. Applying a low FBS concentration, we could reduce the effect of unidentified serum components on the osteogenic differentiation process.

Examining ALP activity, we observed an increase in activity in all three primary cell cultures. The increase was greater in the control group and lasted longer than in the osteogenic group (Figure 9). It can be concluded that osteogenic differentiation is not limited or determined by ALP activity, i.e. other intracellular and extracellular factors influence the ossification process. Some research groups have found a 2- to 3-fold higher ALP activity in response to osteogenic induction when examining DFSCs *(Felthaus et al, 2014, Vollkommer et al, 2015)*. Other research groups examined PDLSCs and measured a 5- to 7-fold increase from baseline *(Kim et al, 2013, Tang et al, 2014)*. Examining DPSCs, an 11-fold increase was measured at day 21 after an initial small increase *(Wei et al, 2007)*, while others described a steady increase in ALP activity of these cells over 3 weeks of osteogenic induction *(Kunimatsu et al, 2018)*. The variation of results may be due to the differences in composition of the differentiation medium and the discrepancies in the method and time point at which ALP activity was measured.

5.2.2. Mineralization in the late phase of osteogenic differentiation

Like other research groups, we observed intensive mineralization with von Kossa staining (Figure 6A) of DFSC, PDLSC, and DPSC cultures by the end of the third week of osteogenic differentiation *(Morsczeck,Moehl et al, 2005, Nagatomo et al, 2006, Kraft et al, 2010)*. We observed calcium deposit formation already on day 14 (Figure 6B) in contrast to others who only detected it after 3 or 4 weeks *(Seo et al, 2004, Lin et al, 2008, Del Angel-Mosqueda et al, 2015)*. Although we could not determine the chemical

composition of these calcium compounds, we could measure their quantitative changes. Examining PDLSC cultures, Kato et al. also observed the formation of calcium deposits on day 14, but they experienced only a threefold increase in calcium concentration between weeks 2 and 3 *(Kato et al, 2013)* in contrast to the more than ten-fold increase we observed (Figure 6B).

5.2.3. Expression of protein markers during osteogenic differentiation

Among the osteogenic markers, almost all cells were stained positively for ON and BSP already at the beginning of the differentiation and their staining was intensified by osteogenic induction (Figure 7A). Similar results have been published by other research groups investigating DPSC *(Gronthos et al, 2000, Galler et al, 2008, Karbanova et al, 2011)*, PDLSC *(Seo et al, 2004, Hayami et al, 2007, Gauthier et al, 2017)* or DFSC cultures *(Morsczeck, Moehl et al, 2005, Guo et al, 2009, Park et al, 2012)*.

Similarly to other research groups *(Kiraly et al, 2009, Park et al, 2012, Ponnaiyan et al, 2012)*, we have shown that the mesenchymal marker vimentin is expressed on the majority of cells in the control group in all three dental primary stem cell cultures (Figure 7A). However, we have detected vimentin even in the osteogenic groups, despite the ongoing ossification process. To date, a similar observation has only been reported in DFSCs *(Morsczeck, Moehl et al, 2005)*. In light of the current literature, our research group was the first to detect high vimentin level expression in PDLSs and DPSCs after osteogenic induction.

Corresponding to the literature data, all three dental MSC types we examined were nestin-positive at the starting point of differentiation, i. e. in the undifferentiated state *(Coura et al, 2008, Kiraly et al, 2009)*. It was a surprising discovery that nestin positivity remained even throughout osteogenic differentiation (Figure 7A). A similar phenomenon has been described by Karbanova et al. for DPSCs *(Karbanova et al, 2011)* and by Morsczeck et al. for DFSCs *(Morsczeck, Moehl et al, 2005)*. Interestingly, not only dental but also bone marrow-derived MSCs have been reported to show no significant change in nestin marker expression after osteogenic induction (Wong et al, 2014).

The results of the two mesenchymal stem cell markers (vimentin and nestin) suggest, along with other literature *(Ariffin et al, 2017, Lindsay and Barnett 2017, Danielsson et al, 2018)*, that dental mesenchymal stem cells retain their stem cell marker expression during osteogenic differentiation.

Among the immunomarkers we examined, STRO-1 was the odd one out. Immunofluorescent labelling of this marker showed no homogeneous staining in the cell cultures (Figure 7B). At the beginning of differentiation, approximately 10–20% of the cells were STRO-1 positive (Figure 8), which correlates with literature data *(Lindroos et al, 2008, Huang et al, 2009)*.

Lindroos et al. found that 19% of DFSCs were positive for STRO-1, which is almost two times higher than what we measured *(Lindroos et al, 2008)*. In addition, Morsczeck et al. detected an even higher percentage of positive cells, namely 35% *(Morsczeck et al, 2010)*. In contrast, and similar to our findings, Guo et al. measured a value of 9% using flow cytometry *(Guo et al, 2013)*. The estimated STRO-1+ data described for DPSCs is also variable in the literature: we can find 13–22% *(Bakopoulou et al, 2011)*, 14% *(Lei et al, 2014)* and 18% *(Machado et al, 2016)*, due to the differences in test methods. For PDLSCs, a 7% positivity for STRO-1 is reported by Lei et al. *(Lei et al, 2014)*. Consequently, the proportion of STRO-1+ cells in the literature has been reported to be mostly around 10-20%. The fact that they were not cultured in the same medium and measured at different passage numbers may have resulted in further discrepancies. Most research groups measured the proportion of STRO-1 cells before osteogenic induction and then differentiated only the selected subpopulation in the osteogenic direction after sorting by flow cytometry. Our research group was the first to describe the changes in the proportion of STRO-1+ cells during osteogenic differentiation of dental stem cells. As a result of ossification, cells excrete calcium deposits around themselves, thereby these adherent cells are practically fixed to the culture vessel. Consequently, these cells cannot be examined by flow cytometry (which requires cell suspension) – only by immunofluorescent staining followed by image analysis.

In this study, significant differences were detected between the osteogenic and control groups for all three primary cell cultures. In the control groups, the ratio of STRO-1+ cells in DFSC and PDLSC cultures decreased significantly compared to the

baseline. In the osteogenic group however, this cell fraction expanded significantly in all three primary cell cultures, and the increase (compared to day 0) was fourfold in DFSCs and almost threefold in PDLSCs by the end of the third week (Figure 9). It is noteworthy that these two cell types also had more intense mineralization compared to DPSCs (Figure 7) where only a 1.5-fold increase was detected in the proportion of the STRO-1 positive cells (Figure 9). Our results suggest that the STRO-1 protein has a potential role in osteogenic differentiation. This assumption is in line with a study on STRO-1 which demonstrated that DPSCs at high passage numbers (P9) are already committed to the osteogenic direction without any inductive agents *(Yu et al, 2010)*.

5.2.4. Expression of osteogenic marker genes during differentiation

In the following section, I summarize our results obtained at the mRNA level and compare them with the literature. We were the first to simultaneously investigate different steps of osteogenic differentiation with assessment of gene expression levels using three primary tooth-derived cell cultures *in vitro*. Ossification is a multistep process, in the first step of which the Runx2 gene is activated, which regulates the Osx, BSP, and OC genes, among others *(Liu et al, 2019)*. Runx2 and Osx genes are involved in the early, the ALPL gene in the middle, and the BSP and OC genes in the late steps of osteogenesis as described by Hayami et al. *(Hayami et al, 2011)*.

For all three primary cell cultures, we measured a 3- to 4-fold increase in Runx2 gene expression during the first week of differentiation (Figure 10). Similar growth in Runx2 mRNA levels has been measured by others in DFSCs *(Vollkommer et al, 2015)* and PDLSCs *(Li et al, 2019)*, in contrast to DPSCs where no significant growth was measured *(Monterubbianesi et al, 2019)*.

The increase in ALP gene expression (characteristic of the middle stage of ossification) was more than 100-fold in DFSCs and PDLSCs, and more than 1000-fold in DPSCs by day 7 (Figure 10). These values are surprising, as we measured only a 5-fold increase in ALP enzyme activity (Figure 9). We can conclude that the elevation in mRNA level is down-regulated at the level of translation. Regarding PDLSCs *(Li et al, 2019)* and DPSCs *(Monterubbianesi et al, 2019)*, significant increases in ALP mRNA

production were reported by other research groups, but not on as large a scale as in our case. Nevertheless, none of these studies compared the mRNA level and enzyme activity of ALP.

Examining the expression of BSP and OC genes involved in the last phase of bone differentiation, we found an increase only in DPSC cultures but not in DFSC and PDLSC cultures. This is a significant difference between the cell types studied. In DFSCs, a different result from ours has been described: a significant increase in OC mRNA *(Vollkommer et al, 2015)*. In PDLSCs we can find similar results in the literature regarding BSP and OC mRNA levels at day 14 *(Li et al, 2019)*. However, when comparing different studies it should be taken into account that the variations in the osteogenic media and the measurement protocols may lead to different results.

Along with the literature, we also concluded that the expression level of key genes may show changes during the ossification process, but is basically very similar for the different types of dental mesenchymal stem cells (DFSC, PDLSC, and DPSC).

The most important observations during my doctoral research are that the proportion of STRO-1 positive cell fraction increases during osteogenic differentiation, and that the expression of nestin and vimentin proteins does not decrease.

5.3. Effect of chlorhexidine, hydrogen peroxide and chlorine dioxide mouthwashes on periodontal ligament stem cells

Currently, there are numerous mouthwashes on the market with a wide variety of active ingredients (e.g. xylitol, NaF, CPC [cetilpiridinium-klorid]). However, mouthwashes containing H_2O_2 and CHX remain the most common in dentistry. These agents have a strong antiseptic, anti-inflammatory, and analgesic effect.

H_2O_2 possesses not only antiseptic but also hemostatic and whitening effects. Because of the latter property, it is also used in the beauty industry. Once H_2O_2 has entered the cell, oxygen radicals are generated, which destroy proteins, lipids and even the DNA. Consequently, it has an apoptotic effect. Its broad-spectrum antimicrobial activity in Gram-positive bacteria is reduced by their catalase activity *(Russel, 2001)*.

CHX molecules are positively charged and therefore bind with high affinity to negatively charged molecules such as those that are found in bacterial cell walls and surface membranes of animal or human cells. By binding to bacteria, CHX causes their lysis *(Jones et al, 1997).* By adhering to the surface membrane (both in soft and hard tissues) CHX can have a long-lasting effect. A side effect of the treatment with CHX is discoloration of teeth due to the precipitation of proteins from the killed bacteria.

In dentistry, ClO_2 was first used as a sterilizing gas, on one hand to sterilize air in dental offices *(Kuroyama et al, 2010)* and on the other to sterilize the surfaces of dental instruments *(Watamoto et al, 2013).* Its applicability in the oral cavity has been hampered by its volatility, as the concentration of oxygen radicals decreases rapidly after treatment *(Noszticzius et al, 2013).* Prior to the research of Noszticzius Z. et al., many toxic by-products appeared during traditional ClO_2 production. This drawback was eliminated by the new membrane process technique developed at the Technical University of Budapest by which a very clear and more stable ClO_2 solution – marketed as Solumium – could be produced *(Noszticzius et al, 2013)*

The research work, a segment of which I present in my dissertation, is a collaboration of several research groups we have recently been able to join. It has previously been shown that the great advantage of ClO_2 over other antibiotics is that microorganisms are unable to develop resistance to ClO_2 in the classical sense *(Noszticzius et al, 2013).* Alternatively, by converting ClO_2 to a gaseous state, it can also penetrate places where tissues are not directly accessible, such as biofilm or dentinal tubules, and thus it can exert its disinfectant effect there as well *(Herczegh, Ghidan et al. 2013, Herczegh, Gyurkovics et al. 2013).* In this chapter, we compared the cytotoxic effect of ClO_2 with H_2O_2 and CHX solutions by investigating PDLSC cells *(Lang et al, 2021).*

5.3.1. Changes in cell morphology and expression of stem cell markers during cytotoxicity studies

Morphological studies have already shown that H_2O_2 and CHX are more cytotoxic to PDLSCs than hyperpure ClO_2. We observed that upon treatment with CHX and

H_2O_2, a large proportion of cells in PDLSC cultures died and the surviving cells lost their fibroblast-like shape and showed a more rounded morphology (Figure 11). In ClO_2-treated PDLSC cultures, the cells retained their healthy fibroblast-like morphology similarly to the controls (Figure 11).

Immunocytochemical studies have demonstrated that the cells that survived the treatments did not lose their stem cell properties. In both P3 and P7 passages, all three stem cell markers examined (STRO-1, CD90, and CD105) were expressed by cells in both the CHX- and ClO_2-treated groups (Figure 14).

5.3.2. Changes in cell viability during toxicity studies

Several studies described the cytotoxicity of H_2O_2 and CHX at the concentrations used in clinical application not only on bacteria but also on healthy human cells *(Redding and Booth 1991, Mariotti and Rumpf 1999, Chang et al, 2001, Kinomoto et al, 2001, Pi et al, 2007, Nishikiori et al, 2008),* which was also supported by our measurements (Figure 13). In contrast, according to our results hyperpure ClO_2 had only a very mild toxic effect on PDLSCs, but at the same concentration it was toxic to bacteria *(Noszticzius et al, 2013).* Studying the locus effects of biofilm, Zs. Lohinai's research group at the Department of Conservative Dentistry (Semmelweis University) found that ClO_2 has the same, if not stronger bactericidal effect in an approximately 10 times lower concentration than CHX *(Herczegh, Ghidan et al. 2013, Herczegh, Gyurkovics et al. 2013).* We obtained a slightly more than 20-fold higher IC_{50} concentration for ClO_2 than for CHX (Figure 12). Consequently, there is a more than 200-fold gap in the case of ClO_2 between its bactericidal effect and its killing effect on PDLSC.

Observing the influence of aging (passage number), the following conclusions were drawn. H_2O_2 is absolutely toxic to the examined cells regardless of the number of passages (Figure 13). CHX was much less toxic to younger (P3) than to older (P7) PDLSC cultures (Figure 13). Unlike the two other compounds, hyperpure ClO_2 had no negative effect on the growth of any PDLSC culture (either P3 or P7) in either of the two concentrations we tested (Figure 13).

6. CONCLUSIONS

The new results of my dissertation are the following:

0. I was the first at Department of Oral Biology to establish the isolation and cultivation protocol for ***DFSC*** primary cultures, and I found them suitable for osteogenic differentiation.

1. By examining changes in ***cell number***, I demonstrated that DFSC, PDLSC, and DPSC primary cultures also grow continuously in a medium containing 1% FBS. During osteogenic differentiation, ***ALP activity*** was increased to a greater extent in the control group than in the osteogenic group.

2. Comparing the degree of ***mineralization*** of DFSC, PDLSC, and DPSC primary cultures, DFSC showed a significantly higher Ca concentration than DPSC.

3. DFSC, PDLSC, and DPSC cultures showed high levels of ***vimentin*** expression before and after osteogenic induction. The proportion of ***STRO-1***-positive cells was significantly increased in the osteogenic group for all three cell cultures.

4. Comparing the expression of genes regulating different steps of osteogenic differentiation, DFSC and PDLSC primary cultures showed a similar pattern in terms of ***BSP***, ***OC***, and ***ALP***, while DPSC cultures showed significantly higher ***mRNA levels*** than DFSC and PDLSC.

5. I demonstrated that the morphology, viability and immunophenotype of ***PDLSC*** primary cultures treated with hyperpure ClO_2 were unchanged compared to the controls, while H_2O_2 and ***CHX*** were toxic to these cells. Consequently, a hyperpure ClO_2 solution can be proposed as a novel effective disinfectant with reduced side-effects for periodontal treatments.

7. SUMMARY

As a dentist, I have been involved in both basic and applied research related to teeth. In my thesis, I have dealt with primary dental stem cells from three different origins.

It was observed that DFSCs divided at a similar rate after isolation, and exhibited a similar fibroblast-like morphology and immunophenotype as PDLSCs and DPSCs. All three primary cell cultures were successfully differentiated in osteogenic direction in the presence of dexamethasone, β-glycerophosphate and ascorbic acid-2-phosphate (osteogenic groups). However, in the absence of these inductive factors, no calcium deposits were formed (control groups). We measured and visualized the intensity of mineralization and found the following order: DFSC>PDLSC>DPSC.

Both cell number and ALP activity, although in different magnitude, increased in both the control and osteogenic groups in all three primary stem cell cultures. Marker proteins specific to MSCs were expressed in both groups of all three primary stem cells at all investigated time points of measurement. Comparing the three primary cell cultures during the first two weeks of osteogenic differentiation, genes regulating osteogenic differentiation showed mostly higher mRNA levels in DPSCs than in PDLSCs and DPSCs.

In dental practice, out of these three stem cell types, PDLSCs are in contact with oral disinfectants used as dental therapy. Thus, in my thesis I have studied PDLSCs in my applied research topic. For the oral rinses tested on PDLSCs, we demonstrated that H_2O_2 and CHX were toxic to primary stem cell cultures at the concentrations used in the clinic, while hyperpure ClO_2 was not toxic.

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9. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

Published articles that form the basis of the doctoral dissertation

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