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QUANTITATIVE AND QUALITATIVE MINERALIZATION EVALUATION OF DENTAL PULPAL AND PERIODONTAL LIGAMENT STEM CELLS USING OSTEOIMAGE ASSAY – AN IN VITRO COMPARITIVE STUDY

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Abstract:

The aim of this study is to evaluate the qualitative and quantitative mineralization ability of dental pulp and periodontal ligament stem cells using osteoimage assay. Comparing the quantitative and qualitative ability of dental pulp and periodontal stem cells using osteoimage assay. Osteogenic mineralization ability of dental pulp and periodontal ligament stem cells help to regenerate the bony defects in the periapical areas of the teeth. Investigating and comparing weather dental pulp or periodontal ligament stem cells aid in the mineralization ability, the objective of this study. Osteogenic differentiation was induced by osteogenic differentiation medium. MTT assay was performed for cell viability and cytotoxicity concentration under different cell concentrations for both cell lines. Fluorescence microscopy was used to measure the qualitative mineralization and quantitative mineralization was done by spectrophotometer. Results obtained showed periodontal ligament cells had better differentiation properties compared with dental pulp stem cells.

Key Words: Mesenchymal Stem Cells (MSCs), Dental Pulp Stem Cells (DPSCs), Periodontal Ligament Stem Cells (PDLSCs),

Introduction:

The goal of Regeneration in endodontics is to improve or restore the function of the diseased or traumatized tissues of teeth. Tissue regeneration strategies require main components such as stem cells, scaffold or matrix and growth factors.[1] Mesenchymal stem cells have the potential to renew themselves for long periods through cell division and, under certain physiological and experimental conditions, they can be induced to become specialized cells. Stemness is the capability of undifferentiated cells to undergo an indefinite number of replications (self renewal) and give rise to specialized cells (differentiation).[17] In recent years tissue engineering in endodontics has taken over. To regenerate the lost defective structures as same from mesenchymal cells i.e. stem cells. Multilineage differentiation properties of Mesenchymal cells has got there importance in tissue engineering along with other elements. In dentistry these mesenchymal stem cells can be obtained from tooth and its surrounding structures. Though cell- to-cell contact and the production of soluble factors, MSCs can induce other regulatory immune cells. When CD3+ T cells were co-cultured with MSCs the proliferation of T cells decreased while percentage of CD4+ CD25+ regulatory T cells increased.[15] Despite of their ability to induce tregs, their functional part in regeneration aids in success treatment results rather having mixed results of failure.[2]

There are five different types of human dental stem cells that can be extracted from the human tooth[1].

- Dental pulpal stem cells
- Stem cells from exfoliated deciduous teeth
- Stem cells from apical papilla
- Periodontal ligament stem cells
- Tooth germ progenitor cell

Dental pulp is made of both ectodermic and mesenchymal components, contains neural crest cells that displayed plasticity and multipotential capability in regeneration, of lost pulp tissue in trauma or caries teeth.[6] The roots of third molars are often incomplete at age of 18 years, therefore, these teeth contain a conspicuous pool of undifferentiated cells in cell-rich zone of dental germ pulp.[18] Periodontal ligament contains heterogenous cell populations that can differentiate into either cementum forming cells or bone forming cells.[16] Revascularization is the procedure being a recent trend that is overcoming the regular followed procedures like apexification followed by root canal therapy. It is well known that mesenchymal stem cells have ability to differentiate into multiple lineages.

Aim:

- Evaluated the qualitative and quantitative mineralization ability of dental pulp and periodontal ligament stem cells using osteoimage assay.

- Compared the quantitative and qualitative ability of dental pulp and periodontal stem cells using osteoimage assay.

Objectives:

- The osteogenic mineralization ability of dental pulp and periodontal ligament stem cells help to regenerate the bony defects in the periapical areas of the teeth
- Investigated and compared weather both dental pulp or periodontal ligament stem cells aid in the mineralization ability, using osteoimage assay.

Materials Required:

Materials:

- Human dental pulp stem cells (DPSCs) and periodontal stem cells (PDLSCs) were taken from stem cell bank.
- Osteogenic medium (alpha Modified Eagles Medium supplemented with 10%fetal bovine serum and 1%penicillin/streptomycin, 250 mol/L ascorbic acid phosphate, 10mmol/L beta glycerophosphate, and 10nmol/L dexamethasone.
- Osteoimage assay
- Spectrophotometry

Methodology:

This study was carried out by obtaining both dental pulp stem cells and periodontal stem cells from stem cell bank. Cells are plated in 96 well plate. Osteogenic differentiation was induced by osteogenic medium. Osteogenic medium Dulbecco's Modified eagle medium + gluta max + 1g/l D-glucose + pyruvate, 10% FBS, 1% penicillin-streptomycin medium supplemented with 0.1µm dexamethasone, 0.2mM L-ascorbic acid and 10mM glycerol-3-phospate.Which was changed every other day. MSCs differentiation into osteoblasts can be achieved by adding vitamin D_3, ascorbic acid and β-glycerophosphate to the culture medium. Some laboratories use dexmethosone a synthetic glucocorticoid instead of vitamin D_3 .[7] From a tissue engineering stand point, constructing a culture environment that closely mimics the native tissue is desirable to replicate tissue functions in vitro.[10] MTT assay was performed for cell viability and cytotoxicity concentration percentage under different cell concentrations for both cell lines. Optical density values were obtained to measure the $CTC_{50.}$ Osteogenic capacity was measured using osteoimage assay. In this the fluorescent staining reagent that binds to the hydroxyapatite crystals formed on the surfaces of the cells and visualised green in color under fluorescence microscope. Brightness of the color determines the mineralization potential of cells. Fluorescence microscopy was used to measure the qualitative mineralization and quantitative mineralization was done by spectrophotometer.

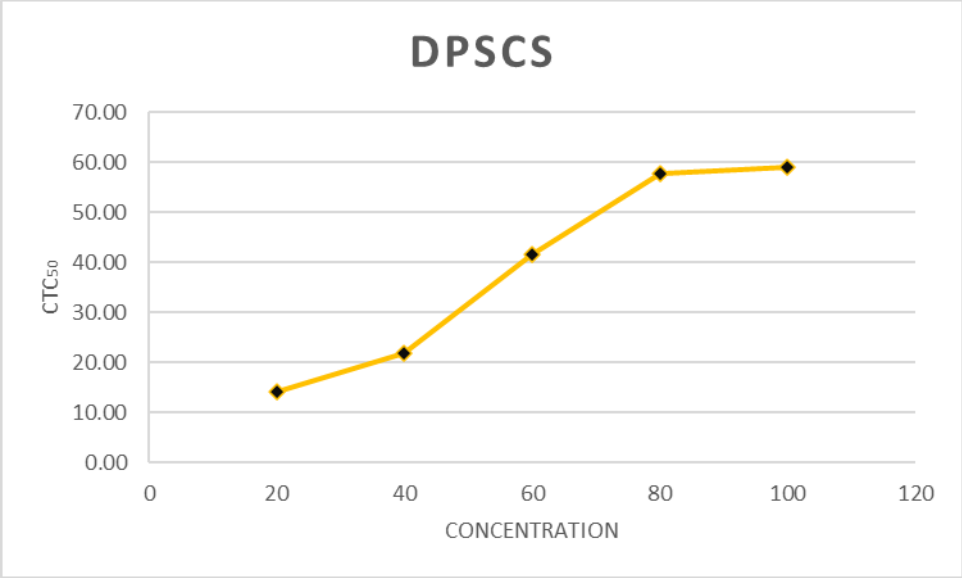
Results:

We determined the osteogenic differentiation abilities of both the cell lines. After 17days of culturing in the osteogenic medium, since our interest was on the osteogenic differentiation of the cells. MTT assay was performed were CTC_{50} percentage values for both the cells were obtained from different cell concentrations, respective graphs are plotted. Spectrophotometer was done to measure quantitative mineralization followed by osteoimage assay under fluorescence microscopy for qualitative mineralization. Control unstimulated cells of both groups showed no changes compared to stimulated groups. Results obtained showed periodontal ligament cells had better differentiation properties compared with dental pulp stem cells.

MTT Assay- Cytotoxicity Assay:

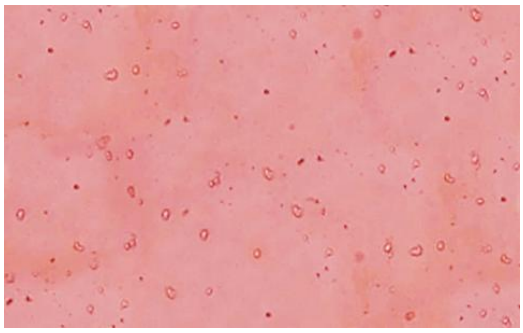
Concentration	Optical Density	CTC_{50}	% CTC_{50}
20	0.417	14.2	77.67
40	0.38	21.81	
60	0.284	41.56	
80	0.205	57.82	
100	0.199	59.05	

Table 1: Cytotoxicity percentage concentration (CTC_{50}) of dental pulp stem cell lines at different concentrations

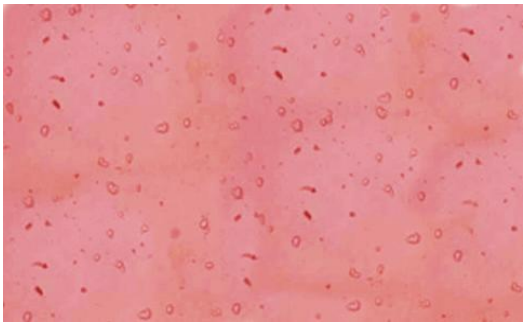


Graph 1: Graph plotted against concentration and the CTC_{50} for DPSCs

20µg/ml



40µg/ml



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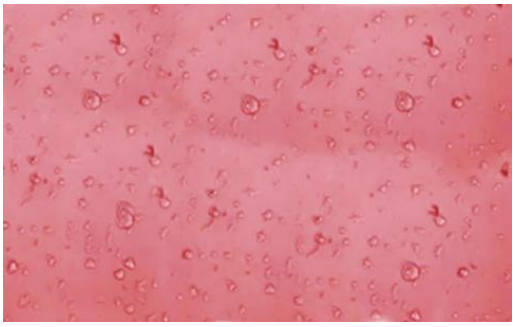
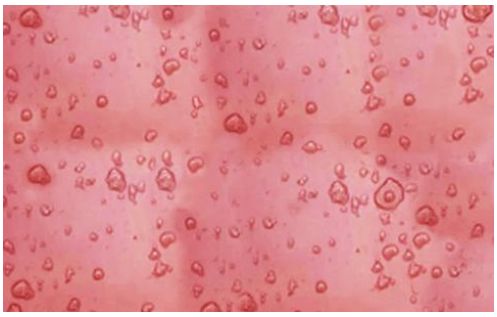


80µg/ml

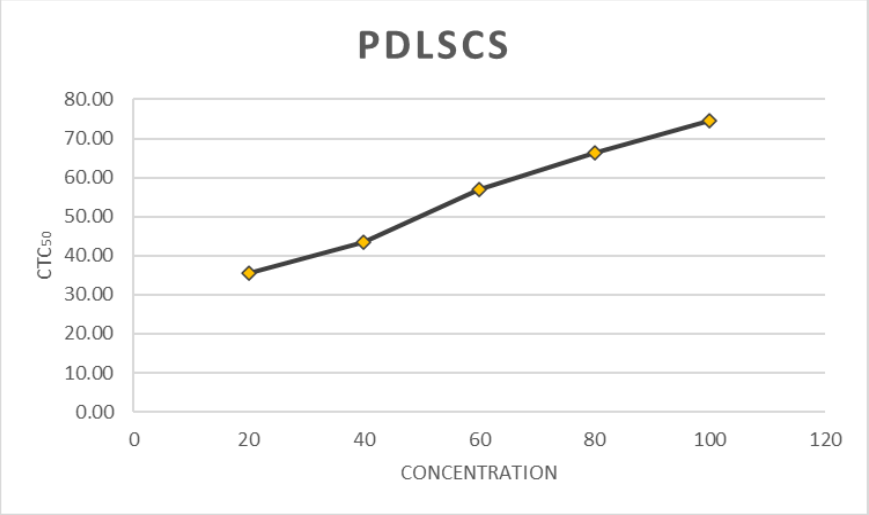


100µg/ml

Picture 1: DPSCs in different concentrations

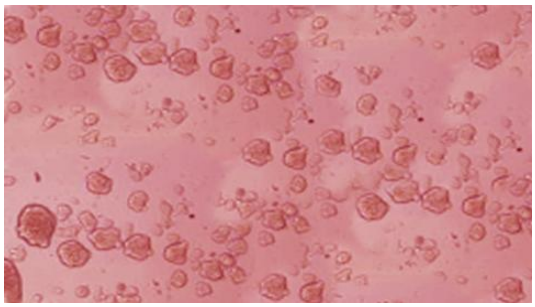
Concentration	Optical Density	CTC_{50}	% CTC_{50}
20	0.314	35.39	49.42
40	0.275	43.42	
60	0.209	57	
80	0.163	66.46	
100	0.124	74.49	

Table 2: PDL SCs cytotoxicity concentration percentage (CTC_{50})

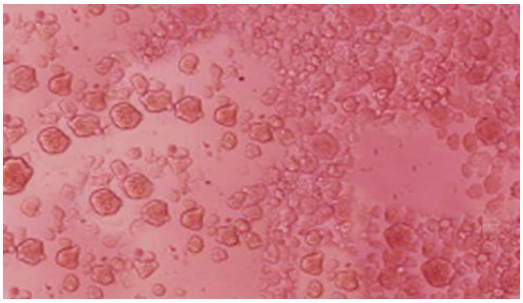


Graph 2: Graph plotted against concentration and the CTC50 for PDLSCs

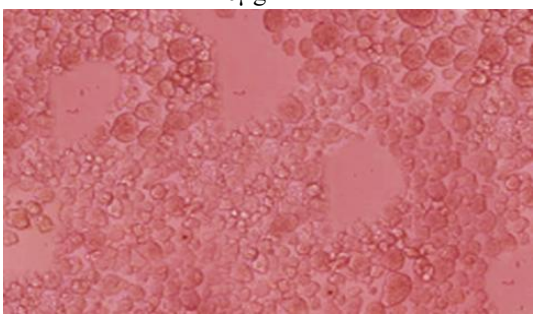
20µg/ml



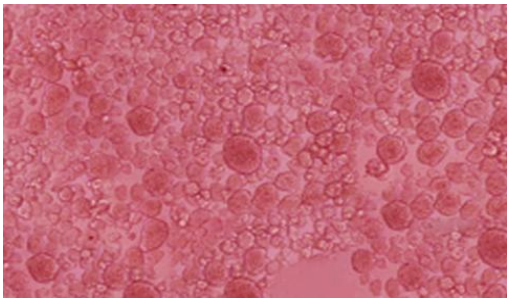
40µg/ml



60µg/m



80µg/ml



100µg/ml

Picture 2: PDLSCs in different concentrations

Days	Triplicate Values (OD)		
	A	B	C
4th Day	0.98	0.98	0.99
7th Day	1.22	1.25	1.27
10th Day	1.37	1.4	1.45
13th Day	2.08	2.09	2
17th Day	2.94	2.95	2.94

Table 3: Triplicate Optical Density values for DPSCs obtained on 4th 7th 10th 13th 17th days

Days	Triplicate Values (OD)		
	A	B	C
4th Day	1.24	1.25	1.26
7th Day	1.58	1.59	1.56
10th Day	1.77	1.74	1.72
13th Day	2.91	2.95	2.98
17th Day	3.99	3.94	3.98

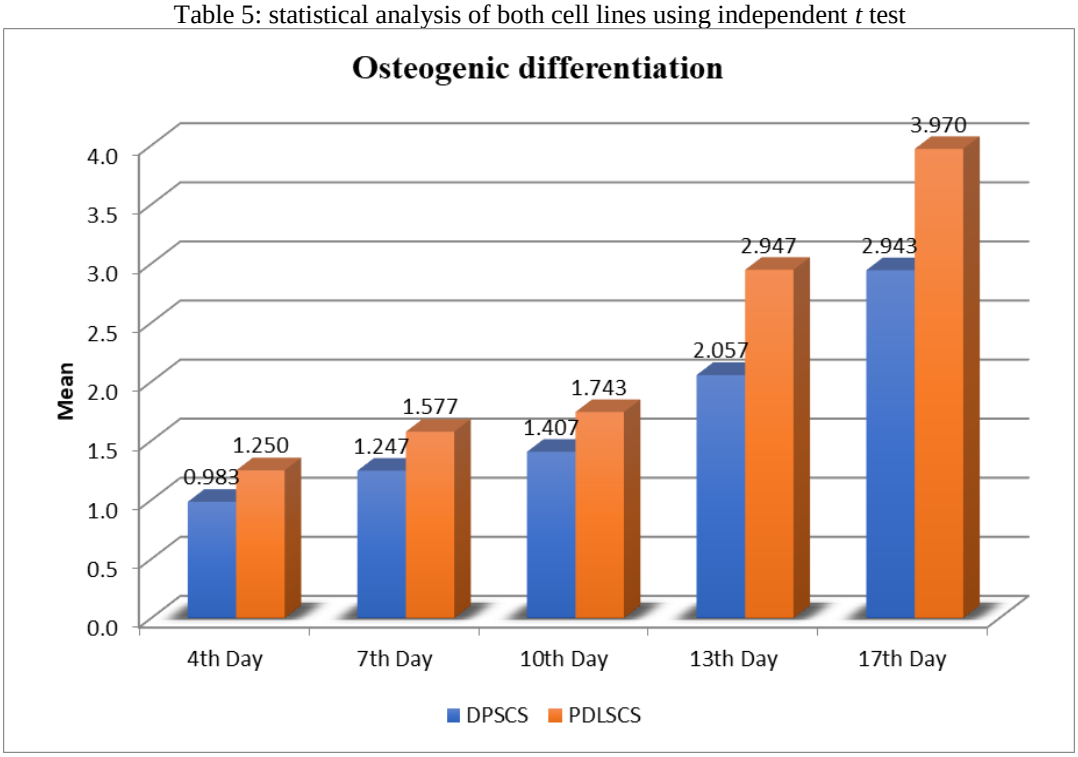
Table 4: Triplicate Optical Density values for PDLSCs obtained on 4th 7th 10th 13th 17th days

Statistical Analysis:

For statistical analyses in this study independent t test was used to determine statistical differences between experimental groups. Statistical significance was set as p value 0.001. It is significant at 1%.

Days	Stem Cell	N	Mean	SD	SE	Independent *t*	*p*
4th Day	DPSCS	3	0.983	0.006	0.003	40	0.001**
	PDLSCS	3	1.25	0.01	0.006		
7th Day	DPSCS	3	1.247	0.025	0.015	19.42	0.001**
	PDLSCS	3	1.577	0.015	0.009		
10th Day	DPSCS	3	1.407	0.04	0.023	12.25	0.001**
	PDLSCS	3	1.743	0.025	0.015		
13th Day	DPSCS	3	2.057	0.049	0.028	25.46	0.001**
	PDLSCS	3	2.947	0.035	0.02		
17th Day	DPSCS	3	2.943	0.006	0.003	65.67	0.001**
	PDLSCS	3	3.97	0.026	0.015		

Table 5: statistical analysis of both cell lines using independent *t* test



Graph 3: Graph plotted between days observed and mean values obtained, from the values of both cell lines on spectrophotometry (Optical Density

Discussion:

Mesenchymal stem cells have the potential to renew themselves for long periods through cell division, However under certain physiological and experimental conditions, they can be induced to become specialized cells. They also show low immunogenicity and an immunosuppressive activity. These features make MSC highly interesting for cell therapy applications. There are five different types of human dental stem cells that can be extracted from the human tooth.[1]

- Dental pulpal stem cells
- Stem cells from exfoliated deciduous teeth
- Stem cells from apical papilla
- Periodontal ligament stem cells
- Tooth germ progenitor cell

Multilineage differentiation properties of Mesenchymal cells has got their importance in tissue engineering along with other elements. These MSCs are capable of giving rise to at least three cell lineages that is osteogenic, chondrogenic, and adipogenic. Other lineages such as myogenic, neurogenic, tenogenic may also derived from bone marrow stem cells. In dentistry these mesenchymal stem cells can be obtained from tooth and its surrounding structures. Their regeneration ability aids in more successful treatment results rather having mixed results of failure.[2]

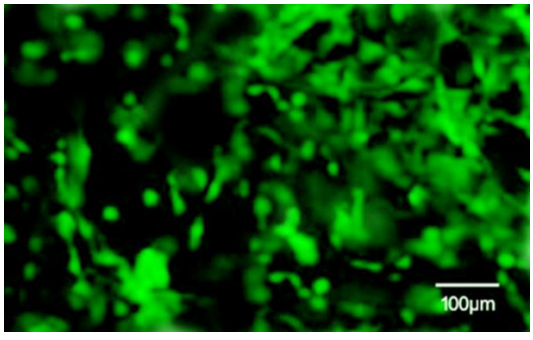
Comparatively with other dental stem cells, dental pulp and periodontal stem cell lines extraction from the tooth is suggestively easier. Functionally, dental pulp is responsible for the maintenance and repair of the periodontal tissue and its associated immune system, has a high regenerative capacity and responds to various types of damage.[5] In the present study choice of the dental pulp and periodontal ligament stem cells has been done.

With tissue engineering, damaged tissues can be repaired via application of stem cells, growth factors and extracellular matrix scaffold. Thus in the present study, dental pulp stem cells was compared with Periodontal ligament stem cells for osteogenic differentiation. which was measured quantitatively and qualitatively.

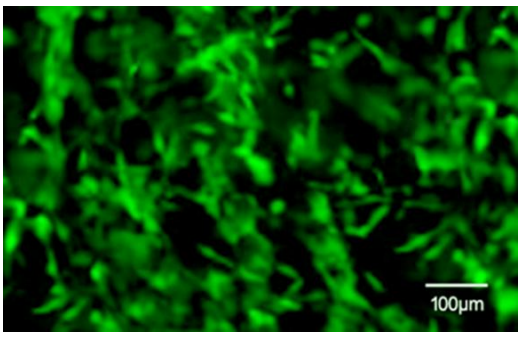
Fluorescence microscopy was used to measure the qualitative mineralization and quantitative mineralization was done by spectrophotometer. MTT assay was performed to observe cell viability. Different concentrations of cells are assessed for cell cytotoxicity concentration (CTC_{50}). Values of percentage cytotoxicity concentration (CTC_{50}) measured (as in Table:1 and Table:2).

DPSCs recorded higher value than PDLSCs cytotoxicity concentration. Percentage Cytotoxicity concentration (CTC_{50}) is fifty percentage cell concentration observed in different concentrations obtained in table 1 and table 2. Osteogenic differentiation was measured with osteoimage assay by fluorescent staining and spectrophotometry.

In this the fluorescent staining reagent that binds to the hydroxyapatite crystals formed on the surfaces of the cells and visualised green in color under fluorescence microscope. Brightness of the color determines the mineralization potential of cells.



DPSCs



PDLSCs

Picture 3: Fluorescent microscopy of cells revealed the fluorescent staining reagent in the assay could only bind to hydroxyapatite clusters formed in both the groups.

Values of spectrophotometry measured for both cell lines, expressed values for Periodontal stem cells (Table:4) were upregulated compared with Dental pulp stem cells. Were unstimulated cell lines did not show any expression. Osteogenic differentiation of periodontal stem cells is better than dental pulp stem cells. Multiple comparisons between stimulated groups showed significant difference p value as 0.001. There is already evidence that there are significant variations in the differentiation potential of single colony derived populations isolated from dental pulp.[5]

Previous studies state that periodontal stem cells could differentiate into periodontal ligaments, alveolar bone, cementum, peripheral nerves. following osteogenic induction, the inhibitory effects of PDLSCs on T cell proliferation was found to be intact.[3] Alveolar bone derived PDLSCs were compared with

conventional root surface-derived PDLSCs and had higher proliferative ability, as well as stronger osteogenic and adipogenic differentiation potential.[3] And the presence of multiple cell types within the postnatal periodontal ligament suggests that these cells may share common ancestors.[8] Subsequently, the optimal choice of MSCs for regeneration of mineralized tissue remains unclear.[14]

In conclusion, this invitro study data demonstrated that periodontal ligament stem cells has shown up-regulation of osteogenic differentiation on respective observations. Periodontal ligaments stems has shown better osteogenic proliferation when observed along with dental pulp stem cells. PDL stem cells could be promising candidates for periodontal tissue engineering. However further studies are needed to understand the exact rationale for the increased proliferative upregulation of periodontal stem cells. And their application in bone regeneration that can help the endodontist restore periapical bony defects by tissue engineering.

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