Tufts University School of Dental Medicine

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Growth and Osteogenic Properties of Dental Pulp Stem Cells from Deciduous vs. Permanent Teeth

Thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

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ABSTRACT

BACKGROUND: Mesenchymal stem cells (MSCs) isolated from dental pulp can differentiate into different cell types including osteoblasts and could therefore be used as a source for autologous bone engineered *in vitro*. In this study, using a 3D culture model, we compared the growth and osteogenic potential of MSCs isolated from human primary deciduous teeth to those isolated from human adult permanent teeth.

MATERIAL AND METHODS: MSCs were isolated and propagated from six healthy permanent and six primary teeth. The colony forming potential (colony forming units (CFU) assay) of these cells was evaluated in non-osteogenic and osteogenic media. Dental pulp MSCs were also embedded in a scaffold material (hydrogel) and grown in osteogenic or non-osteogenic media for 4 weeks. Cell proliferation was evaluated by measuring total cellular DNA using a spectrofluorometric assay. Histology (hematoxylin and eosin) and the amount of mineralization (alizarin red) were evaluated on paraffin-embedded sections. Expression of the differentiation marker genes alkaline phosphatase, osteocalcin, dentin sialophosphoprotein, and bone sialoprotein was evaluated by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

RESULTS: Deciduous dental pulp MSCs formed significantly higher number of CFUs ($p=0.0002$) in DMEM and osteogenic media ($p=0.0351$) and proliferated at a higher rate in osteogenic media ($P=0.0186$) and DMEM ($p=0.0052$) when compared to adult dental pulp MSCs. Compared to adult, deciduous dental pulp MSCs cultured in hydrogel scaffolds showed a higher degree of mineralization and formed larger nodules in both

osteogenic (p=0.0478) and DMEM (p=0.0340). There were no significant differences in the level of expression of all evaluated genes between deciduous and adult dental pulp MSCs. Furthermore, neither cell type was able to completely hydrolyze the hydrogel scaffold.

CONCLUSION: Deciduous dental pulp MSCs has a higher proliferation rate and mineralization potential compared to adult dental pulp MSCs making them an ideal candidate for tissue engineering. However, the hydrogel used in these studies does not seem to be an optimal scaffold for engineering bone-like structures.

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Introduction

Orofacial defects and bone loss can occur congenitally or secondary due to degenerative diseases, tumors, trauma or surgical procedures (Figure 1). These defects can cause problems for functionality of the organ and they can have ever-increasing social impact, especially in the elderly. To date, rebuilding these defects depends on bone grafts from different sources which can cause intrinsic morbidity.[1-2] Autologous grafting to harvest bone and mineralization of hard tissue is one of the common challenges clinicians face when dealing with oral pathologies.[3-4] It is known that bone grafts harvested by surgical procedures can cause high risks of donor site morbidity.[5]

Teeth are hard organs that are susceptible to damage caused by mechanical trauma, chemicals, congenital defects, cancer, and bacterial infections. Teeth do not have the potential to repair or renew damaged structures completely throughout life the way bone tissue, in some circumstances, can. However, adult teeth do show some limited reparative capabilities following injury such as making a protective barrier against dental pulp, also known as tertiary dentin. Tertiary dentin has a poorly organized mineralized matrix in comparison with primary and secondary dentin. When the dentin/odontoblastic layer has been injured following an insult, it is thought that pre-odontoblasts from somewhere within the pulpal tissue migrate to the injured site to produce the protected barrier. However, since these are not mature odontoblasts, the tertiary dentin they produce is poorly mineralized.

The periodontium (PDL, cementum, and alveolar bone) also has a limited potential to regenerate. During orthodontic movement, formation of new cementum and bone and

remodeling of the PDL can be seen, but this can be classified more as a physiological response rather than a true repair or regeneration. In the early phases of periodontal disease there might be slight regeneration of periodontium, but when the disease is established, spontaneous renewal does not happen unless there is some form of therapeutic intervention. Another congenital defect which causes discomfort for patients is restoring alveolar ridges in cleft palate patients. For example, the exfoliation of deciduous teeth in children typically happens between ages 6 to 12 and at the same time restoring the alveolar ridges in cleft palate patients with autologous bone grafting occurs after age 7-8 and this can cause problem on eruption of the teeth, if the bone grafting is not at the right time.

Despite our broad knowledge of the pathophysiology of these diseases, restoration of injured dental tissues so far has relied mainly on the use of artificial implants and structural substitutions which are made of non-vital compounds.[5-6]

1. Stem cells

Any organism, in order to survive, must replace dying cells with new ones. The new cells are generated by cell division; however specialized cells do not themselves divide, but are replenished from stem cells. A stem cell has the ability to self replicate indefinitely during the organism's life, and the right local conditions induce stem cells to specialize. Stem cells have the potential to develop into mature cells that have characteristic forms and specialized functions, such as liver, heart, skin or nerve cells [7] (Figure 2).

There are two kinds of stem cells, embryonic stem cells (ESCs) and non embryonic "somatic" or "adult" stem cells, also called mesenchymal stem cells (MSCs, Figure 2). ESCs are pluripotent stem cells derived from the inner cell mass of an early-stage embryo 4–5 days post fertilization. ESCs are distinguished by their pluripotency, their ability to differentiate into more than 220 cell types, and their ability to replicate indefinitely under defined conditions. MSCs are multipotent, undifferentiated cells found throughout the body after embryonic development that multiply by cell division to replenish dying cells and regenerate damaged tissues.[8-9]

Regardless of their source, stem cells have three general properties: they are capable of dividing and renewing themselves for long periods, they are unspecialized, and they can give rise to specialized cell types.[7, 10] Since obtaining ESCs raises ethical issues, MSCs, which can be isolated from adult tissues, have been recommended for clinical applications.[11]

2. Dental pulp stem cells

Teeth are specialized organs and they do not undertake continuous remodeling the same way that we see in bone tissue. As a result, stem/progenitor cells from dental tissue may be more committed compared to bone marrow-derived stem cells.

MSCs can be isolated from either adult permanent teeth or from deciduous exfoliated teeth (Figure 3). Adult and deciduous dental pulp MSCs are multipotent cells capable of differentiating into various cell types, including osteoblasts, odontoblast, adipocytes, and

neural cells.[12] It has been shown that adult and deciduous dental pulp MSCs can produce irregular and densely calcified structures *in vitro*. They are also capable of forming mineralized tissues similar to dentin when grafted *in vivo*.[4, 13]

In previous clinical trial studies, bone marrow derived MSCs were successfully used instead of autogenous bone grafting for the treatment of osseous defects. However obtaining bone marrow has its own disadvantages. It is an invasive and painful procedure for the donor and the number, proliferation, and differentiation potential of bone marrow derived MSCs decrease with age.[11] The ability of dental stem cells to regenerate organized tooth structures make them ideal candidates to restore damaged structures caused by trauma, cancer, caries or periodontal disease. It is envisioned that stem cell based therapies will help diminish the complications of craniofacial-related surgical procedures requiring allogenic tissue grafts or extraction of autologous bone from secondary sites. This therapeutic approach is a promising choice for bone regeneration and can re-establish bone defects without incurring graft donor site morbidity and allow an effective unlimited source of cellular material.[2, 6]

3. Tissue engineering/regenerative medicine

The goal of tissue engineering is to regenerate tissues based on the combined use of biomaterials and biologic mediators in order to provide new tools for regenerative medicine. Tissue replacement and implementation strategies are specifically needed in the oromaxillo-facial field.[1]

There are two ways tissue engineering is performed: one is by stimulating the body to regenerate tissue on its own and the other way is to make the tissue outside of the body and then use it as an implant to replace a natural tissue .[14] This may be achieved either by transplanting cells into a porous material or a scaffold which has open pores or by relying on ingrowths of cells into such a material, which in both cases develops into normal tissue[13, 15] (Figure 4).

The modern concept of tissue engineering emerged in the late 1980s and one of the key components of this modern concept is the utilization of synthetic biodegradable materials as a scaffold to hold *ex vivo*-expanded tissue cells. The scaffold provides a 3D environment for cells to attach and grow, therefore mimicking the *in vivo* condition. In addition, these synthetic matrices can be fabricated such that they may form any desired shape and carry required growth factors to guide the process of cell differentiation and tissue formation. Generally, tissue-engineering technology involves generating tissue or organ constructs *in vitro* for subsequent implantation.[16]

Successful engineering of functional tissues requires 3D scaffolds that can provide an optimum microenvironment for tissue growth and regeneration.[4] The importance of scaffold materials and design for tissue engineering has long been recognized. Scaffold porosity, biocompatibility and biodegradability, the ability to support cell growth, and use as a controlled gene and protein-delivery vehicle are all highly significant properties for scaffolds.[17]

Hydrogels have recently attracted increasing attention as tissue engineering scaffolds for repairing and regenerating tissues.[18] There is a wide variety of natural and synthetic

materials currently being employed to create such material.[2, 18-22]. It is for these specific reasons that we elected to use hydrogel in this study.

4. Rationale

Dental pulp appears to be an ideal alternative and more readily available source of stem cells. Adult and deciduous dental pulp MSCs have been identified as new sources of stem cells that have the capability of self renewal and multi-lineage differentiation. Previous studies have shown that adult dental pulp MSCs are able to differentiate into odontoblastic and osteoblastic lineages and to produce dentin/pulp complex and bone.[11]

It has been reported that deciduous dental pulp MSCs have higher proliferation rate and almost double cell population in comparison with stem cells obtained from permanent teeth.[23] An advantage for using deciduous dental pulp MSCs over adult dental pulp MSCs is that they can be obtained noninvasively from deciduous teeth which are normally exfoliated or extracted in childhood and generally discarded as medical waste without any ethical concerns. Therefore deciduous dental pulp MSCs might be a better source for tissue engineering.[11, 24]

Due to their potential use for tissue engineering, banking deciduous dental pulp MSCs provides a guaranteed matching donor (autologous transplant) for life. Banking deciduous dental pulp MSCs saves cells before natural damage occurs, it is simple and painless for both the child and parent and since these are adult stem cells they are not subject to the same ethical concerns as embryonic stem cells.[25] While cord blood stem cells have proven valuable in the regeneration of blood cell types, deciduous dental pulp

MSCs are able to regenerate solid tissue types that cord blood cannot, such as potentially repairing connective tissues, dental tissues, neuronal tissue and bone.[2, 20-22, 25]

In this study, we compared the growth and osteogenic potential of deciduous dental pulp MSCs to that of adult and tested the ability of hydrogel to provide a scaffolds to generate bone-like structures.

AIM

The aim of the present study was to compare the growth and osteogenic potential of dental pulp mesenchymal stem cells (MSCs) extracted from deciduous teeth to that extracted from permanent (adult) teeth.

To address this aim we:

1. Extracted, cultured, and expanded dental pulp MSCs from deciduous and permanent teeth

2. Compared their colony forming unit (CFU) potential

3. Compared their proliferation rate in osteogenic and non-osteogenic media

4. Evaluated the osteogenic activity by measuring the gene expression of alkaline phosphatase (ALP), osteocalcin, dentin sialophosphoprotein (DSPP) and bone sialoprotein (BSP) by Reverse Transcriptase Polymerase Chain Reaction (PCR).

5. Compared the histology of the cells cultured in osteogenic and non-osteogenic media using hematoxylin and eosin staining and measure the amount of calcification using alizarin red staining

Study Hypothesis

Deciduous dental pulp MSCs will have higher proliferative and osteogenic properties when compared to adult dental MSCs.

Material & Methods

1- Cell culture

The study protocol was approved by Tufts University Health Sciences Campus. Human dental pulp tissues (6 deciduous teeth and 6 permanent third molars) were obtained from caries free teeth and subject were healthy patients. After extraction, the teeth were immediately placed on ice in Hank's balanced salt solution (HBSS) (Invitrogen, CA) and transferred to the laboratory. Tooth surfaces were rinsed multiple times with phosphate buffered saline (PBS, containing in mM: 145 NaCl, 7.3 Na_2HPO_4, and 2.7 NaH_2PO_4 at pH 7.2), and then the tooth was wrapped in gauze and smashed. Once the tooth cracked and the pulp was exposed, pulp tissue was carefully extracted with fine tweezers. The tissue was digested for 1 hour at 37°C in 0.4 mg/ml collagenase (Worthington, NJ) and 0.2 mg/ml dispase (Sigma-Aldrich, MO). Cell suspension was filtered through a 40 µm cell strainer, and then the cells cultured in Dulbecco's modified eagle's medium DMEM/F12 medium (Invitrogen, CA) containing 10% FBS, 100 µM ascorbic acid, 2mM L-glutamine, and 100 µg/ml penicillin/streptomycin. Cells were grown under routine culture conditions of 95% air and 5% CO_2 at 37°C. The cells were passaged by trypsinization of confluent cells with 0.05% trypsin in 0.53 mM EDTA (pH 7.4) or trypsin replacement.

2- Colony Forming Units (CFU) Assay

Cells were seeded at very low density (1,000 cells/mm^2) and cultured in DMEM/F12 or in osteogenic media. The media was changed every 2 days. After 4 weeks of culture, the cells were washed twice with PBS to remove non-adherent cells and fixed for 20 minutes with 10% formaldehyde made in PBS. After 2 washes in PBS, 0.05% methyl violet was added to each dish for 20 min with gentle shaking. Dishes were washed three times with distilled water and air dried. The number of stained colonies from each cell line was counted in a blinded manner.

3- Cell proliferation

Cell proliferation was measured using a spectrofluorometric assay with Picogreen dsDNA quantitation kit (Invitrogen, CA) that measures the amount of cellular DNA. Cells were mixed with hydrogel (Glycosan BioSystems, UT) and cultured in 96-well plates (Fisher, PA) for 4 weeks. The hydrogel was scooped out from the wells and transferred to a clean 1.5 ml tube containing 250 µl of cell lysis buffer (0.2% v/v Triton X-100, 10 mM Tris-HCl (PH 7.0), and 1mM EDTA). The samples were then frozen in -80°C overnight and processed through a total of two freeze/thaw cycles. After the final thaw, the scaffold was broken up with a pipette tip to improve cell lysis. The amount of DNA in the cell lysate was determined according to the manufacturer's recommendations. After 5 minutes of incubation, fluorescence was measured at 538 nm excitation and 495 nm emission in a spectrofluorometer. The DNA concentration in each sample was determined using a DNA standard curve.

4- RT-PCR

Cells were mixed with hydrogel and cultured in 96-well plates for 4 weeks. The hydrogel was scooped out from the wells, homogenized in TRIzol reagent (Invitrogen, CA) and total RNA was isolated according to the manufacturer's instructions. Twenty nanograms of purified total RNA were used for reverse transcription and PCR amplification with the OneStep RT-PCR Kit (QIAGEN, CA).) using primers specific to alkaline phosphatase (ALP), osteocalcin (OC), dentin sialophosphoprotein (DSPP), bone sialoprotein (BSP) or G3PDH (readymade primer) (Table 1) in a thermal cycler (Applied Biosystems 2720 Thermal Cycler). Primers for ALP, OC, DSPP and BSP were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) according to the mispriming library.

The reverse transcription reaction was conducted at 52°C for 30 minutes followed by PCR according to the manufacturer's instructions. The cycling conditions were 5 minutes hot start at 95°C, 25-30 cycles of denaturation for 40 seconds at 94°C, annealing for 40 seconds at 53°C, and extension for 1 minute at 72°C and a final extension at 72°C for 10 minutes. Samples with no RNA served as the negative controls. After amplification, the products were separated by electrophoresis on a 1.5% agarose gel and visualized by UV light after ethidium bromide staining. Pictures of the gels were digitally captured and analyzed using ImageJ software (http://rsbweb.nih.gov/ij/). The level of expression of each gene was normalized to that of the house keeping gene G3PDH.

Table 1: *Sequences of primers used for RT-PCR*

Gene	Accession No.	Primer Sequence	Amplicon, bp	T_A
ALP	NM_01632.4	**FW: 5'-** CCACGTCTTCACATTTGGTG-**3'**	196	60
		RV: 5'- AGACTGCGCCTGGTAGTTGT-**3'**		
DSPP	NM_014208.3	**FW: 5'-** AGAGGACACCCAGAAGCTCA-**3'**	162	60
		RV: 5'- TGCCTTTCCCAACTTCTTTG-**3'**		
BSP	NM_004967.3	**FW: 5'-** CAACAGCACAGAGGCAGAAA -**3'**	248	60
		RV: 5'- CGTACTCCCCCTCGTATTCA - **3'**		
OSC	NM_199173.4	**FW: 5'-** GACTGTGACGAGTTGGCTGA -**3'**	562	60
		RV: 5'- GCCCACAGATTCCTCTTCTG -**3'**		
G3PDH	NM_008084.2	**FW: 5'-**ACCACAGTCCATGCCATCAC-**3'**	452	59
		RV: 5'-TCCACCACCCTGTTGCTGTA-**3'**		

ALP, alkaline phosphatase; DSPP, dentin sialophosphoprotein; OSC: osteocalcin, G3PDH, glyceraldehyde-3-phosphate dehydrogenase; FW, forward; RV, reverse, T_A, annealing temperature

5- Histology

Cells embedded in hydrogel and cultured in inserts for 4 weeks in DMEM/F12 or osteogenic media were fixed overnight at 4°C in 10% formaldehyde made in PBS. Each insert was then washed twice with PBS for 20 minutes. The hydrogel was removed from the insert and the edges were stained with a green dye (to improve visibility during processing). Samples were then processed for paraffin embedding and 10 µm sections were prepared. From every sample, 16 slides were prepared, each containing 3-4 sections. Paraffin sections were deparaffinized and rehydrated using graded alcohols. For histology

experiments, paraffin sections were processed for hematoxylin and eosin staining. For mineralization experiments, slides were processed for alizarin red (Antec, KY) staining following manufacturer instructions. Slides were viewed under a light microscope (Nikon, Tokyo, Japan) and images were digitally captured using a SPOT camera (Diagnostic Instruments, Inc, MI). To quantify the degree of osteogenesis, the number of alizarin red stained nodules was counted from 3 slides for each sample and the numbers averaged.

6- Data analysis

The primary comparison for the study is deciduous vs. adult teeth in the osteogenic media. Based on other studies, we anticipate that deciduous teeth will have a 3-fold increase over adult teeth (75 vs. 25) and assume a standard deviation of 25. Assuming n=6 per group, we will have 94% power to detect this difference, if alpha = 0.05 (nQuery Advisor, 7.0). The data are expressed as means ± SEM. The data were statistically analyzed using two-sample *t*-test. Values of $p < 0.05$ were considered to be significant.

Results

1. Colony Forming Units (CFU) Assay

In a first series of experiments, we compared the colony forming potential of cells derived from deciduous teeth to that of cells derived from adult teeth and cultured in either osteogenic or non-osteogenic media. As shown in Figure 5A, when cultured in non-osteogenic media, cells from deciduous teeth formed a mean number of colonies (58 ± 3.28) that was nearly twice that of adult teeth (30.0 ± 4.86). This difference was statistically significant ($p = 0.0002$). Similarly and as shown in Figure 5A, when cells were grown in osteogenic media, cells from deciduous teeth formed more colonies (60.3 ± 7.36) compared to cells from adult teeth (32.8 ± 9.43). This difference was also statistically significant ($p = 0.0351$).

These results suggest that stem cells derived from deciduous teeth have a higher proliferative rate than cells derived from adult teeth.

As shown in Figure 5, the number of colonies formed by stem cells derived from deciduous teeth and cultured in osteogenic media (60.3 ± 7.36) was not statistically different from that of cells cultured in non-osteogenic media (58 ± 3.28). Similarly, the number of colonies formed by stem cells derived from adult teeth and cultured in osteogenic media (32.8 ± 9.43) was not statistically different from that of cells cultured in non-osteogenic media (30.2 ± 4.86) (Fig. 5).

These results suggest that the osteogenic media does not alter the colony forming abilities of either deciduous or adult dental pulp stem cells.

2. Proliferation Rate of MSCs Cultured in 3D Scaffolds.

Figure 6 shows that deciduous dental pulp MSCs invaded the hydrogel matrix and formed more extensive networks compared to adult dental pulp MSCs, in either osteogenic or DMEM media.

The data in Figure 7A shows that, in both osteogenic media (138.3 ± 40.9) and DMEM (104.8± 14.19), deciduous dental pulp MSCs had higher proliferative rate compared to adult dental pulp MSCs: (21.9 ± 7.28, osteogenic) and (41.6 ± 13.47, DMEM). There was a statistically significant difference between deciduous and adult dental pulp MSCs in DMEM ($p = 0.0052$) and osteogenic media ($p = 0.0186$, Fig 7A). The type of media did not affect the proliferation rate of either dental pulp MSCs (Fig. 7B) which is in accordance with the CFU data.

Taken together, our data show that deciduous dental pulps MCSs have a higher proliferation rate suggesting that they might be a better choice for tissue-engineering purposes.

3. RT-PCR Analysis of Osteogenic Gene Expression

As shown in Figures 8 and 9, culture of MSCs in osteogenic media did not consistently result in an upregulation of the expression level of any of the genes tested, although that of ALP and DSPP was close to being statistically significant. The mean expression level of ALP gene in osteogenic media for deciduous and adult dental pulp MSCs were 1.08 ± 0.27 and 1.06 ± 0.07, respectively. The data showed the difference was not statistically significant for deciduous ($p = 0.07$) or adult ($p = 0.69$) dental pulp MSCs between DMEM and osteogenic media. The mean expression level of DSPP gene in osteogenic media for deciduous and adult dental pulp MSCs were 0.86 ± 0.08 and 0.77 ± 0.03, respectively. The difference was not statistically significant for deciduous ($p = 0.06$) or adult ($p = 0.08$) dental pulp MSCs between DMEM and osteogenic. The mean expression level of BSP gene in osteogenic media for deciduous and adult dental pulp MSCs were 1.04 ± 0.06 and 0.88 ± 0.09, respectively. The difference was not statistically significant for deciduous ($p = 0.12$) or adult ($p = 0.42$) dental pulp MSCs between DMEM and osteogenic media. Finally, the mean expression level of osteocalcin gene in osteogenic media for deciduous and adult dental pulp MSCs were 0.98 ± 0.04 and 0.90 ± 0.08. The difference was not statistically significant for deciduous ($p = 0.17$) or adult ($p = 0.054$) dental pulp MSCs between DMEM and osteogenic media.

4. Histology

As shown in Figure 10, H&E staining did not reveal major differences in cell morphology between dental pulp MSCs from deciduous or adult teeth. Furthermore, both types of MSCs were unable to completely hydrolyze the hydrogel.

The tissues were processed for alizarin red to determine the degree of osteogenic differentiation between the two cell types. Overall, deciduous (70.4 ± 7.24) and adult (40.7 ± 7.63) dental pulp MSCs cultured in osteogenic media showed greater number of calcium nodules compared to those cultured in DMEM (Figures 11 and 12A). The difference was statistically significant in osteogenic media for both deciduous ($p = 0.0011$).and adult ($p = 0.0111$) MSCs. Furthermore and as shown in Figure 12B, deciduous dental pulp MSCs produced statistically significantly higher number of calcium nodules compared to adult dental pulp MSCs in both osteogenic (70.4 ± 7.24) ($p = 0.0478$) as well as DMEM (8.89 ± 0.49) ($p = 0.0340$).

Taken together, these results show that compared to adult dental pulp MSCs, those from deciduous teeth have a greater osteogenic potential making them an ideal choice for tissue engineering.

Discussion and Conclusions

Orofacial defects and bone loss that can occur congenitally or secondary due to degenerative diseases, tumors, trauma or surgical procedures remain a significant health problem. If unmanaged, it can have an ever-increasing social impact, especially in the elderly. To date, rebuilding these defects depends on bone grafts which can cause intrinsic morbidity.

Bone marrow-derived MSCs have been effectively used in clinical trial for the treatment of osseous defects. However, bone marrow aspiration for preparation of MSCs is an invasive and painful procedure for the donor. Furthermore, these cells tend to lose their plasticity with increasing age. Hence the need for other sources of MSCs for use in tissue engineering and regenerative medicine purposes. An advantage for using deciduous dental pulp MSCs over adult dental pulp MSCs or bone marrow-derived MSCs is that they can be obtained noninvasively from deciduous teeth which are normally exfoliated or extracted in childhood.

The ability of dental pulp stem cells to regenerate organized tooth structures makes them ideal candidates for tissue engineering. Previous report showed that dental pulp stem cells are multi-potent and clonogenic cells, showing a high proliferation potential, and are able to self-maintain for a long period of time. It has also been reported that dental pulp stems cells have the capacity to differentiate into osteoblasts and produce woven bone chips after in vivo transplantation. In another study it was reported that

certain environmental cues (such as 2D versus 3D scaffolds) can enhance the differentiation process of dental pulp MSCs into osteogenic lineages.

In the present studies, we present evidence showing that deciduous dental pulp MSCs possess higher proliferative rate and osteogenic potential when compared to adult dental pulp MSCs. Using the CFU assay, we showed that dental pulp MSCs cells from deciduous teeth formed twice as many colonies as cells extracted from adult teeth. Measuring cell proliferation rate corroborated the CFU data as deciduous dental pulp MSCs proliferated at a higher rate compared to adult cells in DMEM and osteogenic media. Furthermore, compared to adult dental pulp MSCs, deciduous MSCs formed almost twice as many mineralized nodules. These findings confirm and extend those published in the literature.

Another aim of our studies was to test the potential of hydrogel use as scaffolds in 3D cultures to form bone-like structures. The hydrogel used in these studies was Glycosan BioSystems (Extracel-X Maxi-Hydrogel Kit) and is composed of Glycosil™ (thiol-modified sodium hyaluronate), Gelin-S™ (thiol-modified gelatin) and Extralink™ (disulfide-containing polyethylene glycol diacrylate). Solutions of Glycosil and Gelin-S form a transparent hydro-gel when mixed with Extralink [18]. Dental pulp MSCs were embedded (encapsulated) in the hydrogel and cultured for 4 weeks. We found that although our cells did somewhat proliferate within the hydrogel; they did not grow to their full potential. Nevertheless, we found that dental pulp MSCs cultured in osteogenic

media formed mineralized nodules and that cells from deciduous teeth outperformed those from adult teeth.

The lack of full growth in hydrogel could be explained by nutrient diffusion problem in which the availability of nutrients in centrally positioned cells can decrease proportional with the distance to the surface, resulting in starvation of the cells leading to their death. In 2D cultures, all cells have equal access to nutrients from media. Another explanation might be that the cells are lacking other vital components. Indeed, several cell types are dependent upon specific ECM components for growth and differentiation; it is therefore possible that dental pulp MSCs did not fully grow in the hydrogel because they were lacking other ECM proteins. According to the manufacturer, although Gelin-S provides basic cell attachment sites for the cells, ECM proteins (e.g., laminin, collagen, fibronectin, etc) can be easily incorporated non-covalently into the hydrogel prior to gel formation [18]. Similarly, hydrogel could also be supplemented with growth factors known to be important for osteogenesis. These hypotheses will need to be tested in future experiments.

When cultured in osteogenic media, MSCs regardless of their origin are expected to upregulate the expression of genes involved in osteogenesis, such as those examined in the present studies: alkaline phosphatase (ALP), osteocalcin, dentin sialophosphoprotein (DSPP), and bone sialoprotein (BSP) [8, 21-22]. Although mRNA expression levels for ALP and DSPP were upregulated, but not statistically significantly, we could not measure a consistent upregulation of these genes.

It is possible that the culture period was too long or too short to detect changes in gene expression or we missed the peak when the expression of these genes was upregulated. It is also possible that, although the mRNA level did not change, the expression of the target protein did. This hypothesis could be directly tested using either western blotting or immunohistochemistry.

In summary, our results show that deciduous dental pulp MSCs possess higher proliferative rate and osteogenic potential when compared to adult dental pulp MSCs. They also show that the hydrogel-based scaffold used in our studies is not the ideal choice for generating in vitro tissue engineered bone-like material using dental pulp MSCs. Further studies testing the potential benefits of supplementing the hydrogel scaffolds with growth factors and/or ECM proteins for proper osteogenic differentiation of dental pulp MSCs are warranted.

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Figure 1: Schematic showing the different types of bone defects (drcindylayport.com)

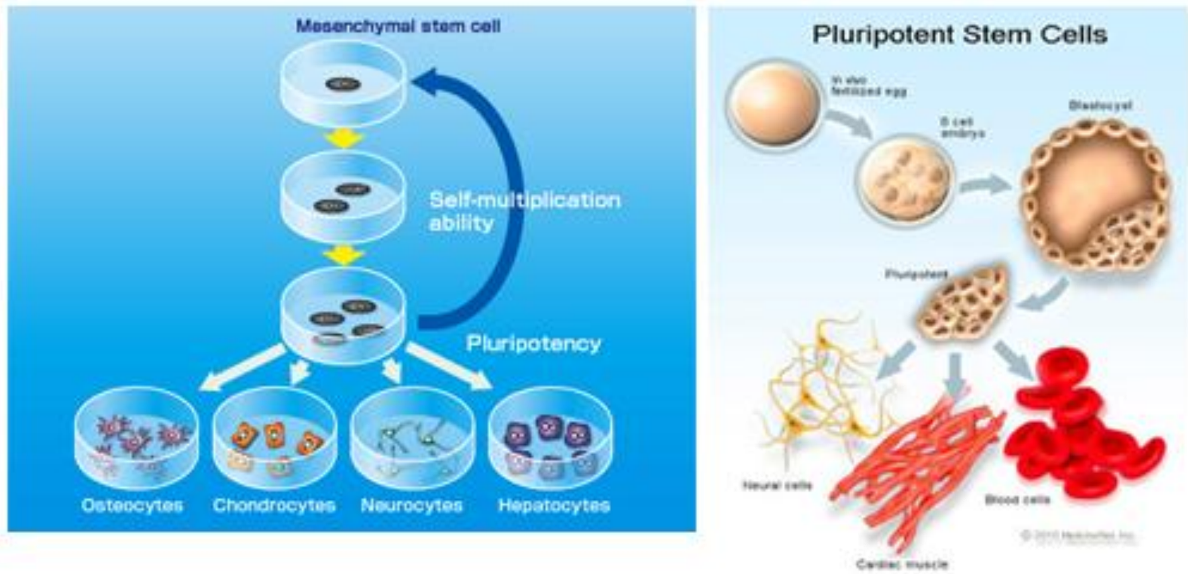

Figure 2: Mesenchymal versus embryonic stem cell (healthsynergyrx.com)

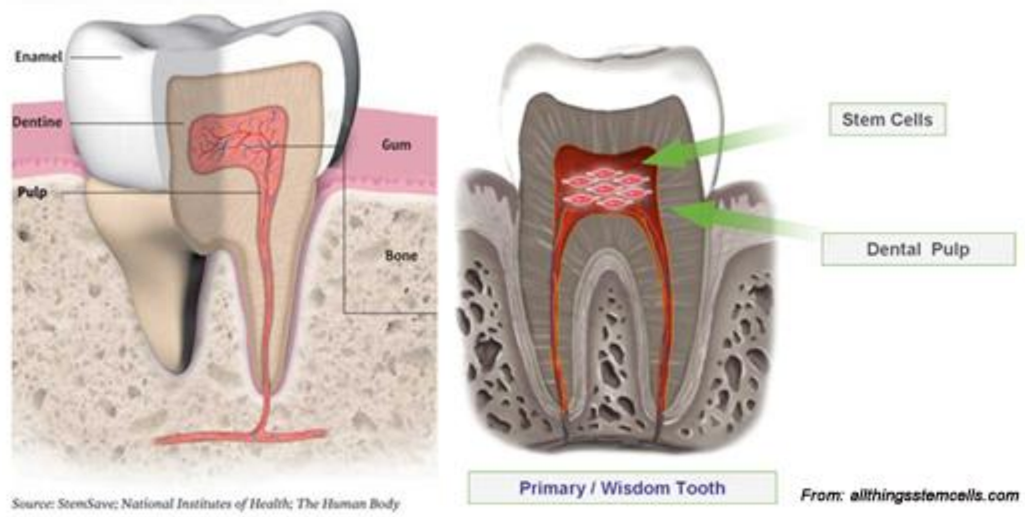

Figure 3. Schematics of tooth structure and location of dental pulp stem cells.

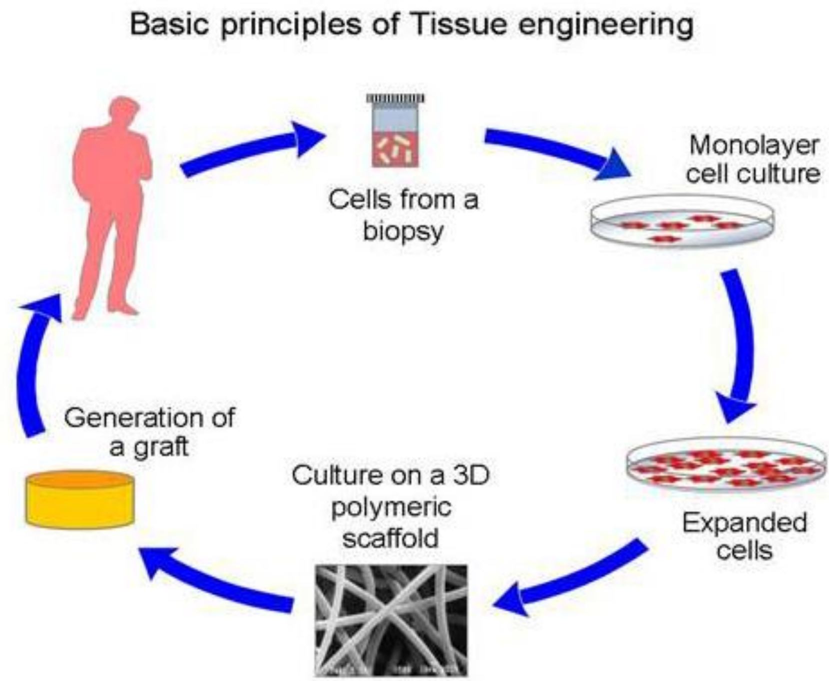

Figure 4. Schematic of the basic principles of tissue engineering (webold.iitd.ac.in)

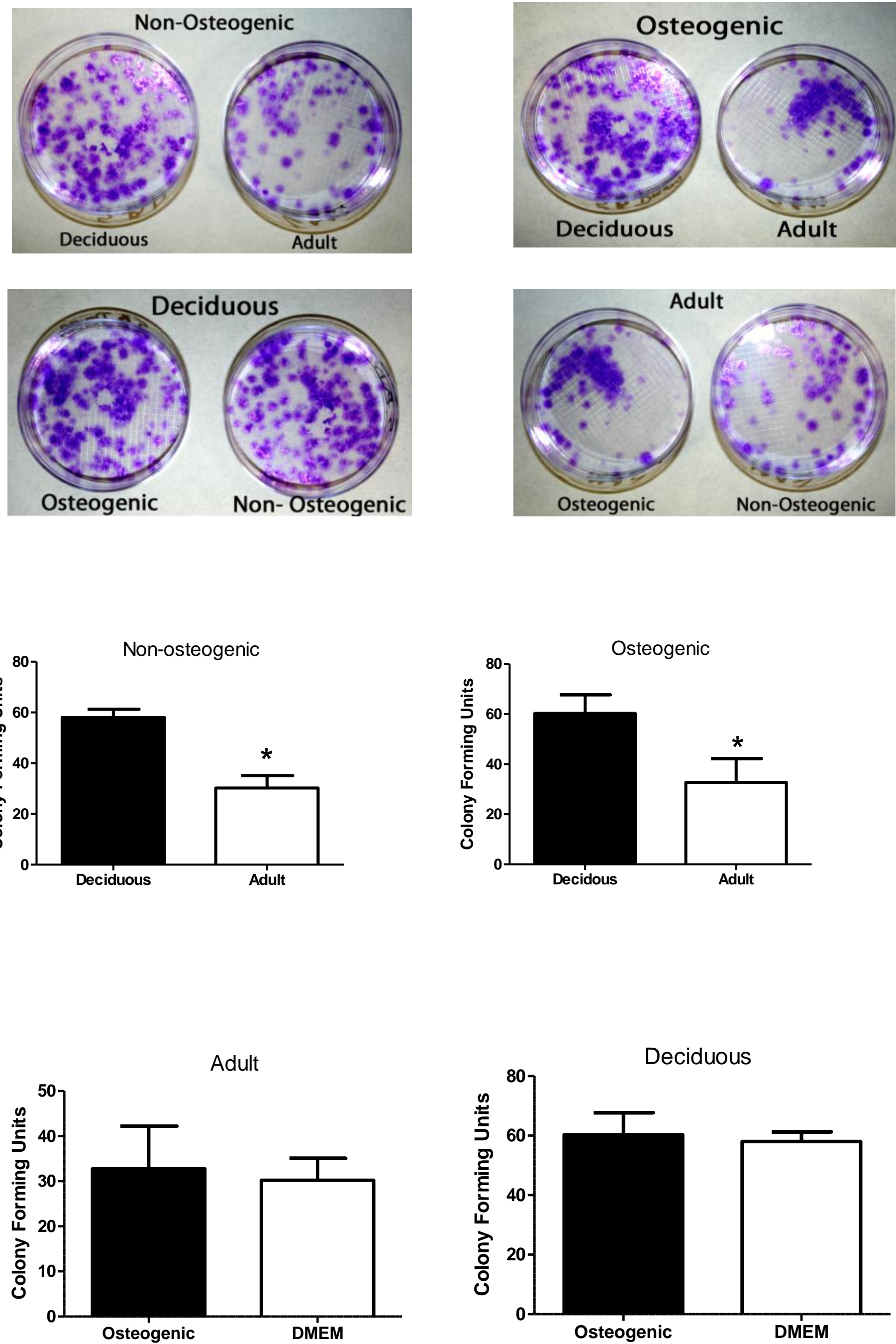

Figure 5: CFUs of deciduous versus adult dental pulp MSCs. Cells were cultured for 4 weeks in DMEM (non osteogenic media) or in osteogenic media. Dishes were stained with crystal violet and the number of CFUs counted. Data in the plots are means ± SEM (n=3). *p<0.05.

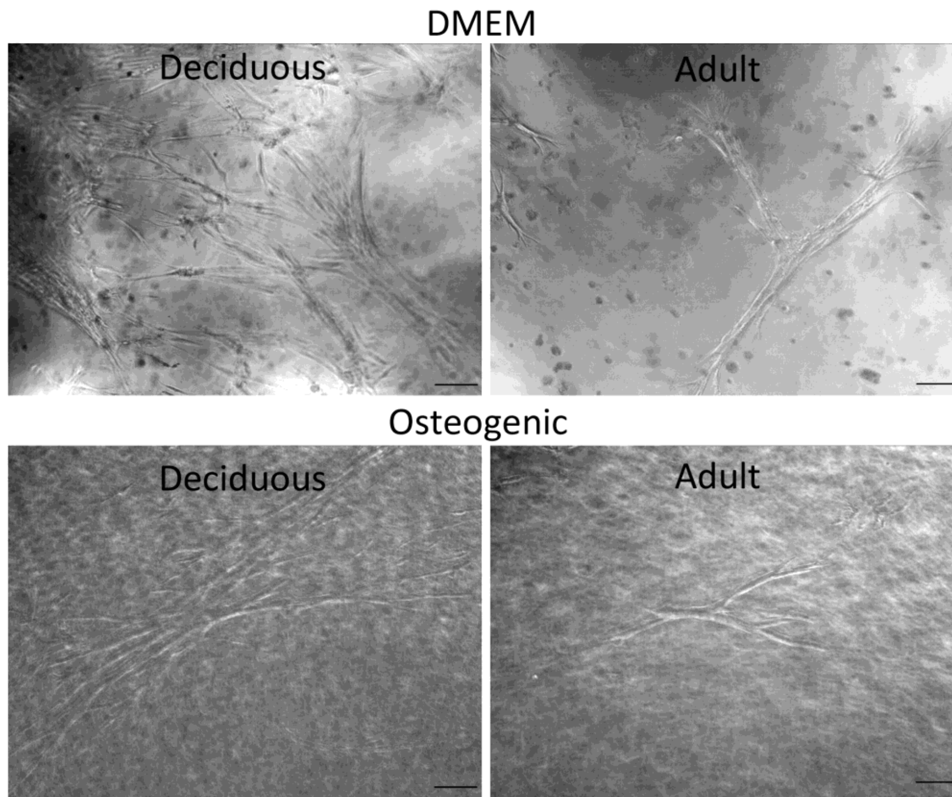

Figure 6: Photomicrographs depicting the morphology of dental pulp stem cells cultured in3D scaffolds. Scale bars=100µm

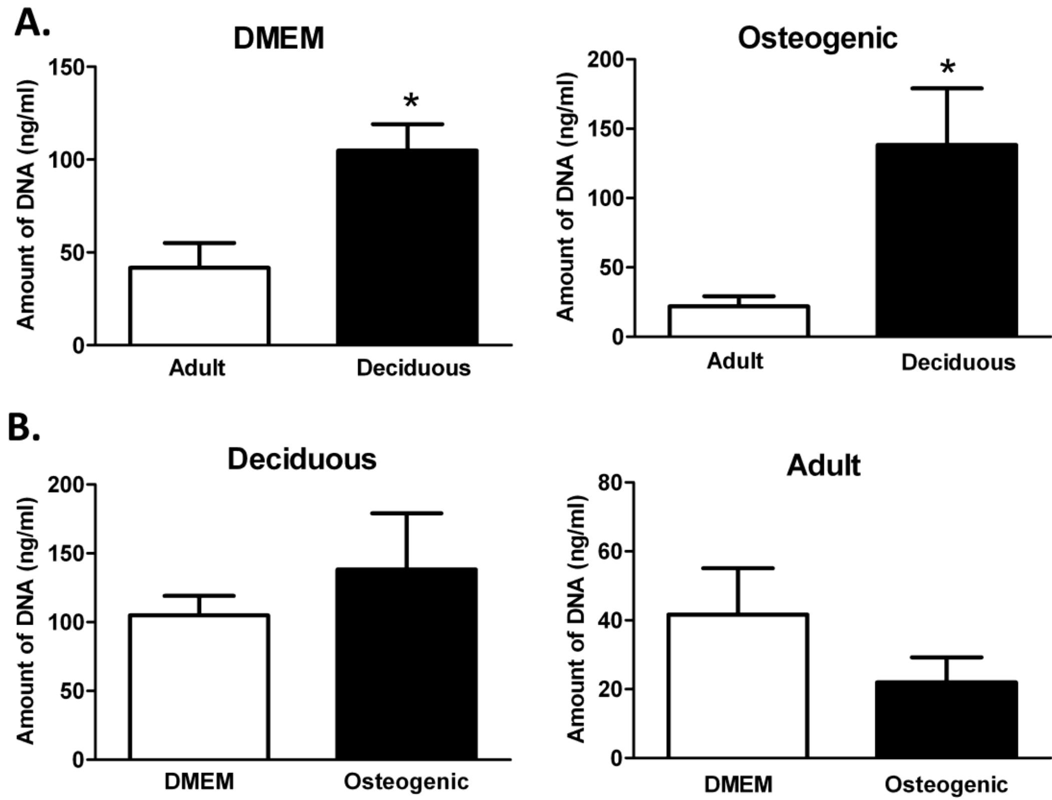

Figure 7: Cell proliferation rate of dental pulp stem cells. Cells were cultured in 3D scaffolds for 4 weeks in either DMEM or osteogenic media. Data are means ± SEM (n=6). *p<0.05.

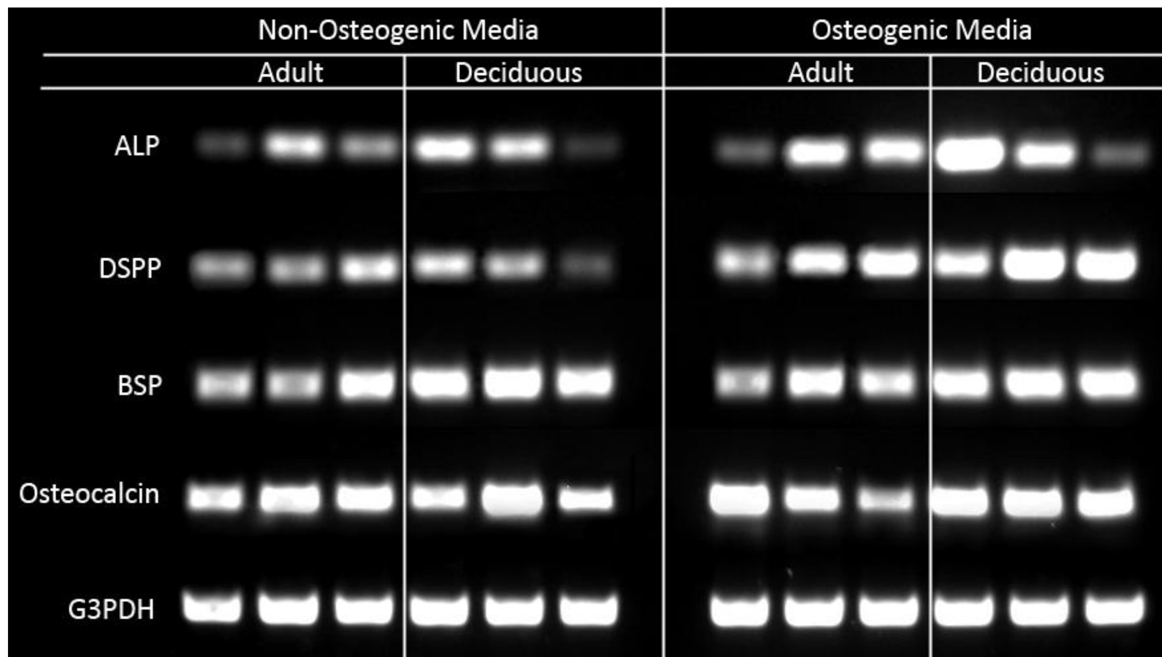

Figure 8: Representative photographs showing mRNA levels of alkaline phosphates (ALP), dentin Sialophosphoprotein (DSPP), bone Sialoprotein (BSP) and osteocalcin (OC). Cells were embedded in hydrogel and cultured for 4 weeks in osteogenic or non-osteogenic (DMEM) media. RNA was extracted and RT-PCR performed. Each lane represents an individual sample.

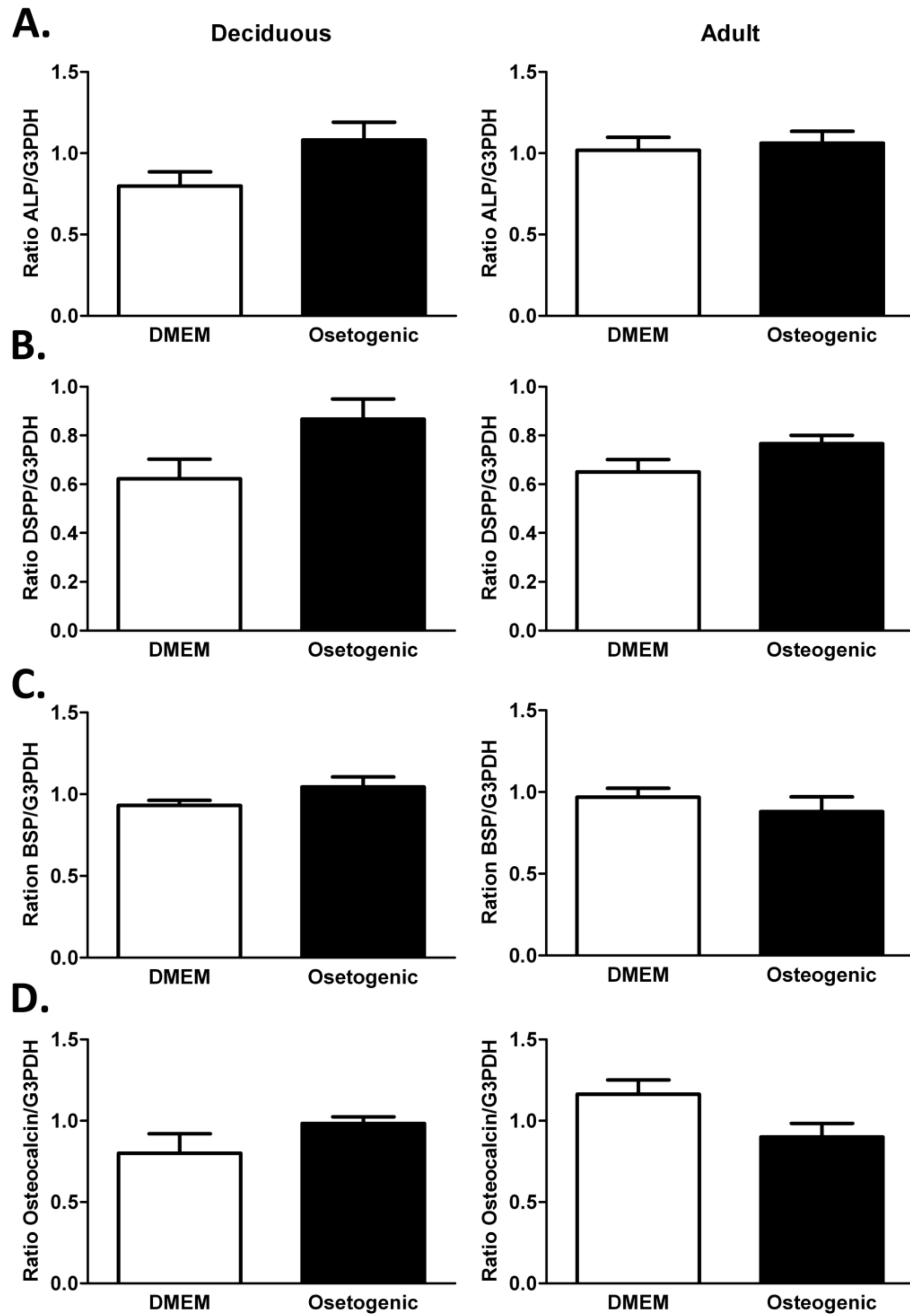

Figure 9: Quantification of the expression level of alkaline phosphates (ALP, **A**), dentin Sialophosphoprotein (DSPP, **B**), bone Sialoprotein (BSP, **C**) and osteocalcin (OC, **D**) mRNAs. Data are means ± SEM (n=6).

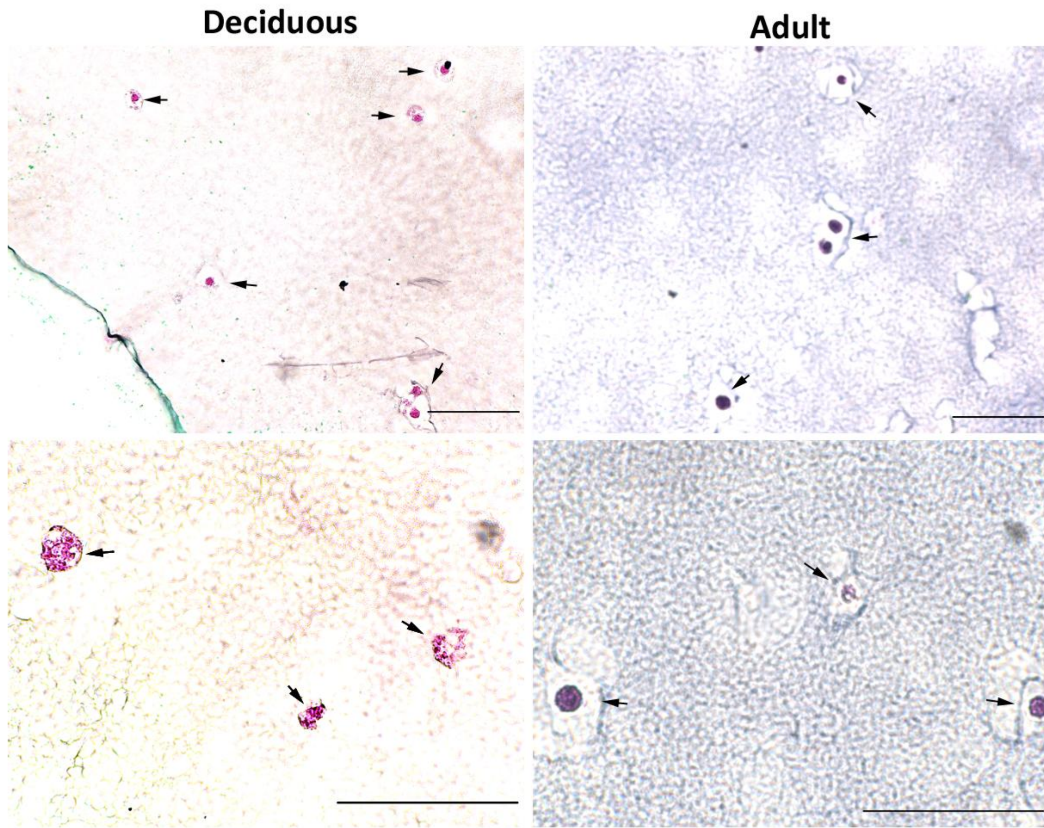

Figure 10: H&E staining dental pulp MSCs from deciduous and adult teeth. Cells were embedded in hydrogel, cultured for 4 weeks in osteogenic media, fixed and processed for H&E staining. Cytoplasm of osteoblasts is basophilic due to the presence of a large amount of rough endoplasmic reticulum. Scale bars =100 μm

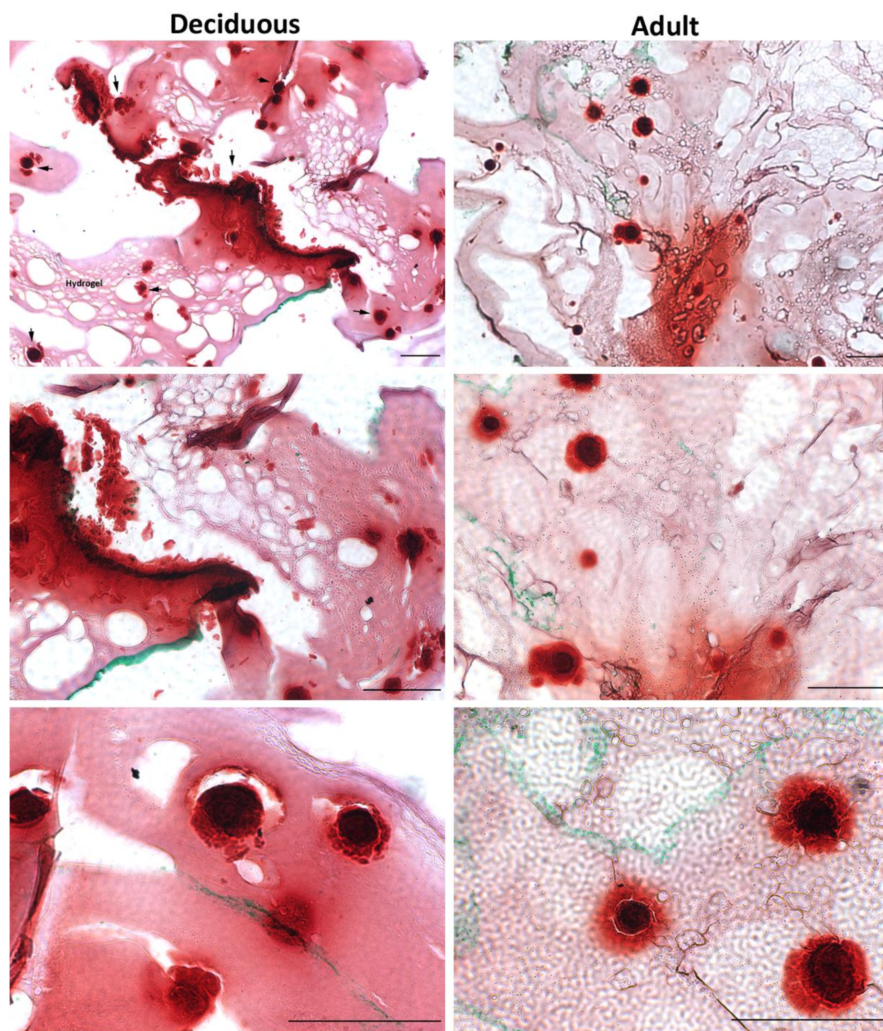

Figure 11. Representative photomicrographs of alizarin red stained sections of deciduous and adult dental pulp MSCs cultured in osteogenic media. Arrows indicate examples of positively stained nodules. Scale bars =100 μm

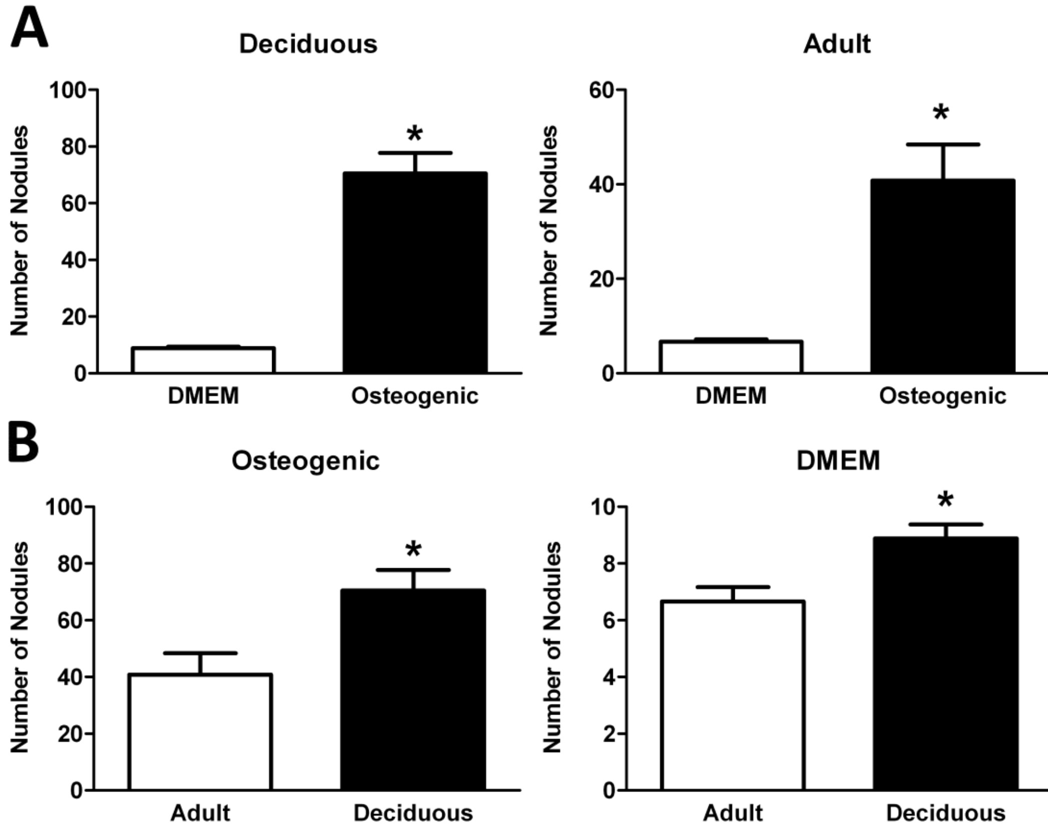

Figure 12: The number of calcium nodules on tissue sections was counted from 3 slides for each cell line and in each culture condition (DMEM vs. osteogenic). Data means ± SEM (n=3). *p<0.05.