UNIVERSIDADE FEDERAL DE PELOTAS
Faculdade de Odontologia
Programa de Pós-Graduação em Odontologia

Tese de Doutorado

Engenharia Tecidual aplicada à regeneração pulpar: Análise da influência das porosidades de um *scaffold* sobre a proliferação e diferenciação odontoblástica de DPSCs.

Ac. Pg. Marcus Cristian Muniz Conde

Pelotas, 2012

MARCUS CRISTIAN MUNIZ CONDE

ENGENHARIA TECIDUAL APLICADA À REGENERAÇÃO PULPAR: ANÁLISE DA INFLUÊNCIA DAS POROSIDADES DE UM SCAFFOLD SOBRE A PROLIFERAÇÃO E DIFERENCIAÇÃO ODONTOBLÁSTICA DE DPSCs.

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Odontologia da Universidade Federal de Pelotas, como requisito à obtenção do título de Doutor em Odontologia (Dentística)

Orientadora: Prof. Dra. Sandra Beatriz Chaves Tarquínio
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DEDICATÓRIA

Dedico este trabalho aos meus pais **Justino** e **Elisabeth** pelo amor e confiança depositados em mim ao longo de todos estes anos de minha formação acadêmica e pessoal. São meus exemplos de generosidade, seriedade e integridade. Amo vocês.

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Jumpin' Jack Flash

I was born in a crossfire hurricane
And I howled at my ma in the driving rain
But it's all right, now, in fact it`s a gas
But it's all right, I'm jumping jack flash, it's a gas, gas, gas!

I was raised by a toothless, bearded hag
I was schooled with a strap right across my back
But it's all right, now, in fact it's a gas
But it's all right, I'm jumping jack flash, it's a gas, gas, gas!

I was drowned; I was washed up and left for dead
I fell down to my feet and I saw they bled
I frowned at the crumbs of a crust of bread
I was crowned with a spike right thru my head
But it's all right, now, in fact it`s a gas
But it's all right, I'm jumping jack flash, it's a gas, gas, gas
(Rolling Stones)

A minha namorada e companheira Mariluci Vargas, pelo amor sincero, incentivo, cumplicidade e principalmente paciência nesses anos que se passaram. Obrigado por tudo. Te amo incondicionalmente.

"O nosso jogo é perigos menina.... o nosso jogo perigoso combina"

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Lá vai nosso herói Dr. Pacheco
Com sua careca inconfundível
A gravata e o paletó
Misturando-se às pessoas da vida
Lá vai Dr. Pacheco
O herói dos dias úteis
Misturando-se às pessoas que o fizeram
Formado, reformado, engomado
Num sorriso fabricado
Pela escola da ilusão
Tem jeito de perfeito
No defeito
Sem ter feito com proveito
Aproveita a ocasião

Dr. Pacheco,vai doutorar
Dr. Pacheco, foi almoçar
Do Do Do Do Do Doutor
Do Do Do Do Do Doutor
Pacheco

Perdido, dividido, dirigido
Carcomido e iludido
Tem nos olhos o cifrão
Disfarça na fumaça
e acha graça
Sem saber que a rua passa
Entre a massa e o caminhão

Dr. Pacheco não vai voltar
Dr. Pacheco foi almoçar
Dr. Pacheco não vai voltar
Dr. Pacheco foi almoçar...

Doutor Pacheco – Raul Seixas

Nota Preliminar

A presente Tese foi redigida segundo o Manual de Normas para Dissertações,Teses e Trabalhos Científicos da Universidade Federal de Pelotas de 2006, adotando o Nível de Descrição 4 – Estrutura em Artigos, que consta no Apêndice D do referido manual, página 38.

RESUMO

CONDE, Marcus Cristian Muniz.**Engenharia Tecidual aplicada à regeneração pulpar: Análise da influência das porosidades de um scaffold sobre a proliferação e diferenciação odontoblástica de DPSCs.**2012.Tese de Doutorado115f. – Programam de Pós-Graduação em Odontologia. Universidade Federal de Pelotas, Pelotas, RS, Brasil.

Propriedades físico-químicas e aplicabilidade biológica de materiais para aplicação em Engenharia Tecidual (ET) são de grande interesse e crescente importância no desenvolvimento de inovações na área de biotecnologia. Na odontologia as pesquisas avançam a cada dia e demonstram que no futuro será possível aplicar terapias para regeneração da polpa dental na prática clínica. Essa transição demandará a capacidade de construção de um tecido pulpar que preencha completamente o canal radicular, produza dentina e tenha uma vascularização suficiente para realização das trocas metabólicas teciduais. Para isso avanços ainda precisam acontecer; a padronização de técnicas e materiais que produzam resultados seguros é indispensável para que as terapias baseadas nos princípios da ET possam ser utilizadas em ensaios clínicos em humanos. O objetivo desse estudo foi realizar uma revisão sistemática da literatura para analisar as técnicas baseadas em *Stem Cell-Based Therapy* com potencial de estabelecer a transição das pesquisas laboratoriais para avalições clínicas em um futuro próximo. Após a revisão, algumas lacunas no conhecimento foram claras.. Assim, foi realizado um estudo para avaliar a influência do tamanho de poros de *scaffolds*, a base de PLLA, na proliferação e diferenciação de DPSCs. Para a obtenção de dois diferentes tamanhos de poros (150-250µm e 251-450µm), utilizamos cloreto de sódio como porógeno. Tooth Slices (1 mm de espessura) foram obtidos de terceiros molares humanos recém extraídos. O espaço correspondente à câmara pulpar foi preenchido com o sal, e então coberto com uma solução PLLA. Após a remoção a polimerização do PLLA e remoção do sal com água destilada foram semeadas DPSC (1×10^5 cells) nos *scaffolds* com diferentes porosidades. A proliferação celular foi avaliada após períodos específicos (3, 7, 14 e 21 dias) utilizando o método WST1. Após 21 dias em cultivo, realizamos o isolamento do RNA, do tecido produzido nos TS, utilizando Trizol®. Avaliamos a diferenciação odontoblástica através de RT-PCR através da expressão de marcadores odontoblásticos (DSPP, DMP1, MEPE), normalizados contra o GAPDH. O RNA de odontoblastos humanos foi o controle positivo. As taxas de proliferação celular foram similares nos dois grupos experimentais. Entretanto, após 14 dias de cultivo, as células cultivadas nos scaffolds com maiores porosidades apresentaram uma taxa de proliferação significativamente maior ($p<0,05$). Após 21 dias de cultivo nos TS, as DPSCs expressaram os três MO, independente do tamanho dos poros. Os tamanhos de poros aplicados por nós foram capazes de sustentar a proliferação e diferenciação das DPSC semeadas nos TS.

Palavras chave: Polpa Dentária, Engenharia tecidual, Scaffold, Células Tronco

ABSTRACT

CONDE, Marcus Cristian Muniz. **Tissue Engineering applied to Pulp Regeneration: Assessment of Scaffold pore size influence on proliferation and differentiation of Dental Pulp stem Cells.** 2012. 115p. PhD Thesis– Post-graduation Program in Dentistry, Federal University of Pelotas, Pelotas, RS, Brazil.

Physicochemical properties and biological applicability of materials to be used in Tissue Engineering (TE) have great interest in the development of innovations in biotechnology. In dentistry research incomes every day and clarify the possibility to implement therapies for regeneration of dental pulp in clinical practice in a short time period. Such translation will require the ability to build a pulp tissue that completely fills the root canal dentin and produce appropriate vascularization to perform the metabolic exchanges needed for human tissues. To do it, we need achieve some advances; standardization of techniques and materials, which produce completely safe results, is essential to do the translation from the lab assays to RCT in humans. Based on that, the aim of this study was to perform a systematic review of the literature to analyze the knowledge regarding the importance of the interface between stem cells and scaffolds. Thereafter, we identify some gaps of knowledge in this field, as well as the techniques that have been employed today with potential to establish the transition from laboratory research to clinical. Among the obtained results, we have detected that the scaffold's physical properties, although imperative in determining cellular behavior were, little exploited since the advent of pulp stem cells. So, we carried out a study to evaluate the influence of the pore size on the proliferation and differentiation of Dental Pulp Stem Cells (DPSCs) *in vitro*. In order to obtain two different pore sizes (150-250μm and 251-450μm), sodium chloride was sieved and used as the porogen-inducer. Tooth slices (1-mm thickness) were obtained from recently extracted third molars and after pulp tissue removal, scaffolds with both porogen inducer sizes were prepared using PLLA *(*Poly-L-lactic acid) inside the pulp chamber. DPSCs (1 x 10^5 cells) were seeded in the scaffolds with different porosities, in 24-well plates with specific medium. The cell proliferation was evaluated using the WST1 assay at 3, 7, 14 and 21 days intervals. Also, after 21 days of culture, the RNA of seeded cells was extracted using Trizol and RT-PCR technique was used to assess the differentiation of the DPSCs in odontoblasts, using putative odontoblast markers (DSPP, DMP1 and MEPE). RNA from fresh odontoblasts was used as a control. Cell proliferation rate was similar in both scaffolds except for the 14 days period, when the cells seeded in the scaffolds with larger porosities showed higher proliferation ($p<0.05$). After 21 days DPSCs seeded into the dentin slices expressed the differentiation odontoblastic markers, independently of the pore sizes. The two different pore sizes tested allowed the DPSCs proliferation and differentiation.

Key Words: Dental Pulp, Tissue Engineering, Scaffold, Stem Cells

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Artigo 2: Influence of scaffold pore size on Dental Pulp Stem Cells proliferation and differentiation

Lista de abreviaturas e siglas

%	Percentual
μl/ml	microlitro por mililitro
®	símbolo indicativo de marca registrada
μ	Micron
μl	Microlitro
Acid - Co-CS-HA	*Collagen–Chondroitin Sulfate–Hyaluronic*
BMP-2	*Bone Morphogenetic Protein 2*
BMP-7	*Bone Morphogenetic Protein 7*
$Ca(OH)_2$	Hidróxido de Cálcio
CBB	*Ceramic Bovine Bone*
CDP	Complexo Dentino-Pulpar
CE	Células Endoteliais
cm^3	Centímetros Cúbicos
CO_2	Dióxido de Carbono
DAPI	4',6-Diamino-2-phenylindole
DDM	*Demineralized Dentin Matrix*
DMP1	*Dentin Matrix Protein 1*
DNA	*Deoxyribonucleic Acid*
DPSC	*Dental Pulp Stem Cell*
DSC	*Dental Stem Cells*
DSP	*Dentin Sialoprotein*
DSPP	*Dentin Sialophosphoprotein*
DTE	*Dental Tissue Engineering*
ECM	*Extracellular Matrix*
EDTA	*Ethylenediamine tetraacetic acid*
EP	Entrez PubMed
EUA	Estados Unidos da América
FBS	*Fetal Bovine Serum*
FC	Fatores de Crescimento
FFD	Flávio Fernando Demarco
FGF2	*Fibroblast Growth Factor 2*

FITC	*Fluorescein isothiocyanate*
GF	*Growth Factor*
HÁ/TCP	*Hydroxyapatite/Tricalcium Phosphate*
HDMEC	*Human Dermal Microvascular Endothelial Cell*
HEK293	*Human Embryonic Kidney 293 Cell*
IWS	ISI Web of Science
MCMC	Marcus Cristian Muniz Conde
MEC	Matriz Extracelular
MEM-α*	Meio Essencial de Eagle - Modificação Alfa
MEPE	*Matrix Extracellular Phosphoglycoprotein*
MEV	Microscopia Eletrônica de Varredura
ml	Mililitro
mm^3	Milímetros Cúbicos
MOI	Multiplicidade de Infecção
MMP-2	*Matrix Metalloproteinase-2*
mRNA	*Deoxyribonucleic Acid*
MTA	*Mineral Trioxide Aggregate*
O_2	Oxigênio
ºC	Graus Celcius
OPLA	*D,D-L,L-polylactic acid*
PA	*Peptide-Amphiphile*
PBS	*Phosphate Buffered Saline*
PGA	*Polyglycolic*
pH	Potencial Hidrogenionico
PL	*Platelet Lysate*
PLGA	*Poly-l- Lactic-co-Glycolic Acid*
PLLA	*Poly-l-Lactic-Acid*
RGD	*arginine-glycine-aspartate*
SC-TB	*Stem Cell-Based Therapy*
SFB	Soro Fetal Bovino
SHED	*Stem Cells from Exfoliated Deciduous Teeth*
SIS	*Small Intestinal Submucosa*
SS	SciVerse Scopus

TDM	*Treated Dentin Matrix*
TGF-β1,	*Transforming Growth Factor Beta 1*
TS/S	*Tooth Slice/Scaffold Model*
VEGF	*Vascular Endothelial Growth*

SUMÁRIO

1. PROJETO DE PESQUISA

1. 1 Introdução

Atualmente 85% da população mundial apresenta necessidade de reparos,em diferentes magnitudes, nas estruturas da região craniofacial(SCHELLER; VILLA-DIAZ; KREBSBACH, 2012). Dentre estas desordens a perda dental compreende uma porção significativa e afeta pessoas de diferentes faixas etárias(POLZER et al., 2010).As principais causas de perda dental são a doença periodontal (GAMONAL; LOPEZ; ARANDA, 1998), o trauma dental (ALANI; AUSTIN; DJEMAL, 2012) e a doença cárie.

Na população brasileira necessidade de prótese dental é identificada já aos 15 anos (SB Brasil 2012) sendo que aos 24 anos de idade 29,7% da população já apresenta tal necessidade (CORREA et al., 2010). Neste contexto, dados alarmantes são detectados na população adulta (35 - 44 anos), da qual 68,8% necessitam da reposição de um ou mais dentes (SB Brasil 2010). Apesar de o edentulismo não ser uma condição que represente risco de morte, exerce um impacto crucial na qualidade de vi dados indivíduos afetados.Além do prejuízo na função mastigatória, a perda dental proporciona problemas de ordem nutricional, afeta a fonação e a estética (ROUMANAS, 2009),e proporciona limitações sociais (POLZER et al., 2010). Os materiais sintéticos, restauradores e protéticos utilizados atualmente, apesar de serem de fácil aplicação e possuírem relativo baixo custo, não são capazes de induzir a regeneração da parte afetada- ossos maxilares, dentes, vasos sanguíneos do organismo receptor (FERRACANE; COOPER; SMITH, 2010). Assim, estes materiais proporcionam um reparo limitado da função e estética do tecido original(SMITH et al., 2008).

Uma teoria revolucionária trouxe à tona a possibilidade da regeneração completa de tecidos e até mesmo órgãos; a Engenharia Tecidual foi proposta como um campo interdisciplinar que funde princípios e inovações da Engenharia e das Ciências Biológicas (LANGER; VACANTI, 1993). Este campo da ciência tem por objetivo o reparo ou substituição de tecidos e órgãos baseado em três pilares fundamentais: as moléculas bioativas - Fatores de crescimento (FC), moléculas de origem proteica, com capacidade de se ligarem a receptores específicos presentes

na membrana celular, e a partir daí reger o comportamento dessas estruturas (MATTUELLA et al., 2007); os scaffolds – estruturas tridimensionais que servem como substrato para a adesão e proliferação das células simulando assim a matriz extracelular; e as células-tronco que possuem alta capacidade clonogênica, de proliferação e capacidade de diferenciação em uma ampla gama de tecidos(LANGER; VACANTI, 1993).Na Odontologia, o desenvolvimento de terapias que visam à regeneração ou reparo biológico das estruturas dentais tem produzido um crescente interesse entre os pesquisadores da área (SMITH et al., 2008).

A polpa dental é um tecido conjuntivo de características únicas que possui como função primordial a formação de dentina (BJORNDAL; MJOR, 2001) a partir de uma matriz orgânica secretada pelos odontoblastos (LINDE; GOLDBERG, 1993). Devido à natureza pós-mitótica das células odontoblásticas, a polpa possui uma capacidade de regeneração extremamente limitada (LINDE; GOLDBERG, 1993).Todavia, este tecido possui um requintado mecanismo de defesa que permite o reparo das estruturas do complexo dentino-pulpar – CDP(GOLDBERG; SMITH, 2004). Neste contexto, lesões crônicas desencadeiam eventos moleculares que sinalizam para os odontoblastos intensificarem sua atividade secretória e assim, produzir dentina reacional no local subjacente à injúria(GOLDBERG; SMITH, 2004). Sólidas evidências (GRONTHOS et al., 2002; GRONTHOS et al., 2000; MIURA et al., 2003) demonstram que a polpa, de dentes permanentes e decíduos, possui em seu interior uma população de células mesenquimais progenitoras denominadas "*Dental Pulp Stem Cells* (DPSC)" e "*Stem Cells from Exfoliated Human Teeth* (SHED)", respectivamente. Quando o CDP é submetido a injúrias de rápida progressão, como lesões de cárie aguda ou trauma dental que proporcionam a destruição dos odontoblastos (BJORNDAL; MJOR, 2001) há uma sinalização para que esta população de células progenitoras migre para o sitio da injúria(TECLES et al., 2005). Após o processo de migração essas células se diferenciam em odontoblasto símiles e iniciam a secreção de dentina reparadora(GOLDBERG; SMITH, 2004). Entretanto, o processo de formação de dentina reparadora não se dá de forma espontânea; é necessária a aplicação de materiais sintéticos que estimulem este evento biológico(BJORNDAL; MJOR, 2001).

Tradicionalmente o material utilizado para induzir o reparo do CDP é o Hidróxido de Cálcio – $Ca(OH)_2$ – e mais recentemente o Agregado de Trióxido Mineral – MTA(FORD et al., 1996). Estes produtos induzem a formação de tecido

mineralizado no local da exposição pulpar, e devido ao seu elevado pH possuem propriedades bactericidas e bacteriostáticas. Apesar dos resultados satisfatórios esses materiais não possuem um alvo de ação específico(ALMUSHAYT et al., 2006). Quando em contato direto com a polpa provocam uma necrose superficial que proporciona comprometimento de sua estrutura; o reparo se dá à custa de tecido sadio(HILTON, 2009). Além disso, as terapias vitais da polpa possuem indicações restritas e geralmente seu prognóstico em longo prazo não é promissor (TUNA, 2009). Desta forma, a terapia endodôntica convencional é indicada em situações de envolvimento pulpar, justamente por possuir um prognóstico mais favorável (DEMARCO et al., 2005). Contudo, a excisão total do tecido pulpar apresenta desvantagens consideráveis no que concerne à manutenção da estrutura dental na arcada dentária. O tratamento endodôntico causa uma perda considerável de tecido dentinário tendo como consequência um dente não vital e mais suscetível a fraturas (DEMARCO et al., 2005). Isto é especialmente crítico quando se trata de trauma dental em dentes permanentes de pacientes jovens (NOR, 2006)

A viabilidade e manutenção do órgão dental na arcada dentária estão diretamente relacionadas com a vitalidade do tecido pulpar, uma vez que a polpa vital proporciona nutrição e exerce a função de receber e perceber estímulos patogênicos(BJORNDAL; MJOR, 2001). Além disso, a manutenção da vitalidade pulpar é essencial para a contínua deposição de dentina(BJORNDAL; MJOR, 2001). A interrupção da dentinogênese, tanto nos dentes decíduos quanto nos permanentes jovens, dá origem a características morfológicas que podem complicar a realização de possíveis tratamentos endodônticos, como câmara pulpar volumosa, menor espessura de dentina radicular e formação incompleta da estrutura apical(NOR, 2006). Assim, empregar a engenharia de tecidos para a obtenção de tecido pulpar que permita a completa formação dos tecidos e anexos radiculares é uma estratégia que se mostra cada vez mais factível.

Dentre as células utilizadas em estratégias que visam à regeneração do tecido pulpar destacam-se as SHEDs. Estas células, assim como as DPSCs, são uma população altamente clonogênica e multipotente. Além disso, as SHED possuem uma capacidade superior de proliferação, maior número de duplicações e formação de aglomerados esféricos quando comparadas às DPSC (MIURA et al., 2003). Descobertas recentes têm reforçado a justificativa do emprego das SHEDs nas tentativas de regeneração do tecido pulpar (WANG et al., 2012). Consistentes

estudos demonstraram que quando implantadas no interior do canal radicular, de dentes extraídos de pacientes saudáveis, as SHEDs são capazes de se diferenciar em tecido conjuntivo semelhante ao tecido pulpar (CORDEIRO et al., 2008) com células odontoblásticas capazes de formar matriz dentinária (SAKAI et al., 2010).

Um dos princípios fundamentais que governa a engenharia tecidual diz que o tecido construído (*construct*) deve possuir e manter uma vascularização adequada para suportar seu crescimento (DES RIEUX et al., 2011; SAKAI et al., 2010). O fornecimento de nutrientes e oxigênio para os *constructs* é essencial para sustentar a alta atividade metabólica das células que se dividem, proliferam e diferenciam com a finalidade de formar novos tecidos (DAI; RABIE, 2007). Naturalmente, sem uma rápida formação e difusão de uma rede vascular os *constructs* devem se limitar a uma espessura de até $3mm^3$para evitar morte celular e necrose dos tecidos neoformados (DEMARCO et al., 2011). Outro limitante para a regeneração pulpar é sua localização anatômica, pois é circundada quase em sua totalidade por paredes rígidas mineralizadas e seu aporte sanguíneo advêm somente do forame apical (DEMARCO et al., 2011). É importante salientar que a migração de vasos sanguíneos do organismo hospedeiro em direção ao interior do implante também desempenha papel categórico na viabilidade das células implantadas e consequentemente na neoformação tecidual (GALLER et al., 2012).

A angiogênese, ou neovascularização, é o processo de formação de novos vasos sanguíneos a partir de vasos pré-existentes, com expansão contínua de uma árvore vascular em resposta a um aumento na massa tecidual (FERRARA, 1996). O VEGF (Vascular Endothelial Growth Factor) é uma glicoproteína que desempenha papel central no processo de angiogênese (DVORAK, 2005),pois possui uma atividade mitogênica sobre células de origem endotelial (JABBARZADEH et al., 2008). Este fator pró-angiogênico proporciona a neovascularização de polpas submetidas a traumas (GONCALVES et al., 2007; MULLANE et al., 2008) e pode ser utilizado como agente tópico para o reimplante de dentes avulsionados (MULLANE et al., 2008).Consistentes estudos conduzidos no Laboratório de Angiogênese da Universidade de Michigan (Ann Arbor, Michigan - EUA) demonstraram que as SHEDs são capazes de se diferenciar em células endoteliais com capacidade de formar vasos sanguíneos (SAKAI et al., 2011). Esta capacidade de diferenciação das SHED é de fato de extrema relevância (D'AQUINO et al., 2007). Cordeiro et al. (2008), utilizando a técnica do *tooth slice*(GONCALVES et al.,

2007; SAKAI et al., 2011), concluiu que quando SHEDs são co-implantadas com células endoteliais humanas, formam um *construct* que apresenta uma melhor organização e maior celularidade do que quando apenas SHED são implantadas no dorso de camundongos. No entanto, não se observou diferença significativa no número de vasos sanguíneos formados nas duas condições experimentais testadas - SHED + células endoteliais ou somente SHED. Além disso, foi demonstrado que as SHED foram capazes de se diferenciar em células endoteliais com capacidade de formar vasos (CORDEIRO et al., 2008). Estas informações elucidam que as células-tronco da polpa possuem potencial angiogênico inerente. Também, Sakai et al. (2010) demonstraram que as SHED possuem capacidade de se diferenciar em células endoteliais com capacidade de formar vasos sanguíneos que realizam anastomose com a vasculatura do organismo hospedeiro (SAKAI et al., 2010).

Visto que a vascularização insuficiente dos *constructs* proporciona morte celular e necrose dos tecidos neoformados (DVORAK, 2005), uma ampla gama de estratégias foi desenvolvida para promover a vascularização adequada dos constructs (GOODWIN, 2007). Estratégias utilizando VEGF para estimular a proliferação e migração de células endoteliais ou utilizando células que possam se diferenciar em tecidos de origem endotelial já obtiveram determinado sucesso como exposto anteriormente (CASAGRANDE et al., 2010; CORDEIRO et al., 2008; GALLER et al., 2012; SAKAI et al., 2010). Ambas as abordagens, isoladamente ou em combinação, resultaram em uma melhoria significativa do crescimento vascular, provando a hipótese de que o fornecimento de fatores de crescimento pró-angiogênico desencadeia a migração de CEs hospedeiras (GALLER et al., 2012). Entretanto, existem ainda desafios a serem superados nas técnicas de entrega de fatores de crescimento. As moléculas recombinantes utilizadas são extremamente sensíveis ao processamento térmico e exposição a solventes, possuem uma meia-vida insuficiente quando implantadas *in vivo* e é extremamente difícil prever e controlar sua distribuição temporal e espacial após a inoculação no organismo de interesse (JABBARZADEH et al., 2008; SCHELLER et al., 2012).

O conceito básico da terapia gênica (TG) é derivado de nossa habilidade de inserir material genético no núcleo celular para manipular as proteínas que são produzidas pelo maquinário endógeno de determinada célula (SCHELLER et al., 2012). Desta forma, a TG nos permite realizar a entrega de genes de interesse, em doses fisiológicas por um determinado período de tempo(JABBARZADEH et al.,

2008). Para isso são utilizadas moléculas denominadas de vetores, como plasmídeos e polímeros sintéticos de origem não viral(PARK; JEONG; KIM, 2006). Apesar do baixo risco biológico associado a este tipo de vetores ainda não é possível realizar a transfecção celular com a mesma efetividade dos vetores de origem viral (PARK et al., 2006). Dentre os vetores virais estão incluídos os retrovírus, lentivirus, *herpes simplex* vírus e os adenovírus(SCHOTT et al., 2011). Os adenovírus compreendem uma ampla família de vírus não envelopado que contém uma dupla fita de DNA genômico de aproximadamente 36 Kpb (GRIEGER; SAMULSKI, 2012) com 50 diferentes sorotipos virais identificados. Com a aplicação da TG, genes específicos podem ser transferidos para células alvo de um determinado organismo vivo – Terapia Gênica *In Vivo* em organismos humanos. Entretanto, este tipo de terapia não é comumente realizado devido à dificuldade em transfectar somente as células de interesse e o risco de reação adversa devido à introdução do vetor (CAVAZZANA-CALVO et al., 2000). Como alternativa pode ser aplicada a Terapia Gênica *Ex-Vivo*(TGEx), na qual células são cultivadas e modificadas com a utilização de um vetor contendo o gene de interesse *in vitro*. Depois de transfectadas as células são implantadas em um sítio específico de um organismo (JABBARZADEH et al., 2008).

Na odontologia, a combinação de TGEx com as técnicas de engenharia tecidual possui um grande potencial de aplicação nas estratégias de regeneração do órgão pulpar. A TGEx se mostrou eficiente na melhora da vascularização de *constructs* que foram semeados com células geneticamente alteradas para realizar a secreção de fatores pró-angiogênicos (IWAGURO et al., 2002; JABBARZADEH et al., 2008). Além disso, este tipo de terapia pode ser realizado com células do próprio paciente (transplante autólogo), o que evita problemas imunológicos. As SHED, já citadas anteriormente, são obtidas de um tecido considerado descartável, proveniente de dentes decíduos naturalmente esfoliados (MIURA et al., 2003). Este fácil acesso ao tecido para o isolamento das SHED é fator crucial para superar os obstáculos relacionados à dificuldade de obtenção de células de pacientes adultos para terapias ex-vivo com células autógenas (MURRAY; GARCIA-GODOY; HARGREAVES, 2007). Da mesma forma, estas células são um material com grande potencial para a implementação de Bancos de Células-Tronco (ARORA; ARORA; MUNSHI, 2009) uma importante ferramenta para a realização da transição dos

resultados obtidos em laboratório para a prática clínica- pesquisa translacional (ISASI; KNOPPERS, 2011).

1.2 Objetivo

O objetivo de nosso estudo será investigar a capacidade de proliferação, diferenciação e liberação de VEGF das SHEDs transfectadas com *rhVEGF*$_{121}$, além comparar seu potencial angiogênico e de neoformação de tecido pulpar ao das células tronco não-transfectadas (Miura et al., 2003).

1.3 Objetivos Específicos

- Realizar, *in vitro*, a transfecção e avaliação da viabilidade e proliferação das SHED transfectadas com *rhVEGF*$_{121}$;
- Preparar os Scaffolds Injetáveis (RADA-16),para o cultivo inicial das SHED transfectadas com *rhVEGF*$_{121}$;
- Avaliar a taxa de transfecção ideal no sistema de co-transfecção HEK293 como sistema celular de hiperprodução em diferentes MOIs (0, 5, 10, 20, 50 e 100);
- Avaliar a morfologia celular por Microscopia Eletrônica de Varredura (MEV);
- Avaliar a liberação de VEGF e diferenciação odontoblástica das células *in vitro;*
- Avaliar a atividade mitogênica das SHED transfectadas sobre células de origem endotelial;
- Avaliar a capacidade de diferenciação em linhagens odontoblásticas, *in vitro*, em canais de pré-molares humanos;
- Avaliar a capacidade das SHED transfectadas em produzir tecido pulpar funcional *in vivo*,
- Realizar a análise histológica e imunoistoquímica dos tecidos pulpares produzidos *In Vivo*. A partir destas análises, comparar o tecido formado pelas células transfectadas com o tecido formado pelas células controle.

1.4 Hipótese

A hipótese a ser testada é de que as SHED transfectadas com o gene *rhVEGF*$_{121}$ apresentarão um potencial de indução de angiogênese superior ao das SHED (Miura et al., 2003).

Além disso, avaliamos que as células alteradas geneticamente serão capazes de originar um tecido semelhante à polpa dental com uma vascularização significativamente superior e com melhor organização celular e tecidual do que a gerada pelas SHED.

1.5 Materiais e Métodos

Toda a parte experimental desta pesquisa será realizada na *School of Dentistry* da *University of Michigan*. Todo o material empregado, biológico ou não, será obtido e fornecido pela referida Instituição.

1.5.1. Preparo dos Scaffolds Injetáveis

O RADA-16 (1%w/v, BD Bioscience) será preparado a partir da diluição do peptídeo purificado em uma solução de sucrose a 10% (Sucrose Crystal, J.T. Baker Chemical Co) para a obtenção de uma concentração final de RADA-16 igual a 20% v/v (RADA-16/20). Para promover a ressuspensão das células no scaffold, estas serão dissociadas utilizando tripsina/EDTA 0,25%, lavadas duas vezes em solução de sucrose 10%, contadas e ressuspendidas na solução de RADA-16 previamente preparada.

1.5.2. Cultivo Celular

SHEDs serão cultivadas com meio de cultura MEM-α suplementado com 10% de soro fetal bovino – SFB e 1% de solução de penicilina e estreptomicina (Gibco Penicilin-Streptomycin, Invitrogen) em frascos de cultivo de 75cm^3. O frasco será incubado em uma atmosfera controlada (37ºC, 95% O_2 e 5% de CO_2). Em todos os experimentos serão utilizadas células em estágio de subconfluência (80%) entre as passagens 4 e 6.

1.5.3. Transfecção adenoviral de *rhVEGF$_{121}$* nas SHED

As SHED serão transfectadas com adenovírus utilizando o sistema de co-transfecção HEK293, um sistema celular de hiperprodução, (GRUSS et al., 2003). Vetor Adenoviral dl7001 (Sorotipo 5) com as regiões E1 e E3 deletadas será utilizado neste estudo, utilizando CMV como promotor de transgênese. As SHED em estágio de subconfluência na passagem 3 serão contadas e transfectadas com o adenovírus contendo rhVEGF$_{121}$ em diferentes MOIs (0, 5, 10, 20, 50 e 100). Após 24h de incubação o meio de cultivo contendo a carga viral será removido e as células já transfectadas (*SHED/rhVEGF$_{121}$*) serão lavadas três vezes com solução salina estéril (PBS).

1.5.4. Semeadura das células nos *scaffolds*

Para a ressuspensão das SHED e das SHED/rhVEGF$_{121}$ no RADA-16/20 as células serão dissociadas utilizando solução de tripsina/EDTA a 0,25% (Gibco Penicilin-Streptomycin/EDTA, Invitrogen). Então, as células serão lavadas duas vezes em solução de sucrose 10%, contadas e ressuspensas no RADA-16/20 em uma concentração de (1x10^5 células/ml).

1.5.5. Viabilidade, liberação de VEGF e diferenciação odontoblástica das células *in vitro.*

5.5.1. Viabilidade das células no *scaffold*

Após a ressuspensão das células em RADA-16/20 (1x10^5 células/ml), 50 µL das soluções células/*scaffolds* será injetado em placas de 96 poços. Os conjuntos serão armazenados em estufa a 37°C por 1 hora. Após este período será adicionado 50 µL de meio de cultura a cada poço para obter um volume total de 100 µL. Diariamente, 30 µL de meio de cultura serão renovados em cada poço. A cada 24 horas, uma placa será removida da estufa e 50 µL do conteúdo de cada poço será cuidadosamente removido.

As células serão fixadas com 20 µL de solução de formalina tamponada 10% (Formalin Buffered 10%, Fisher Chemical) durante 15 minutos. Logo após, os 20 µL superficiais serão removidos e 30 µL de PBS serão adicionados e mantidos por 15 minutos para promover uma lavagem e diluição da formalina remanescente. Feita a lavagem os 30 µL superficiais serão retirados para realização da coloração dos filamentos de actina F das células com 30 µL de faloidina 2,5% em PBS (Alexa Fluor 488 phalloidin, Invitrogen, Carlsbad) por 30 minutos no escuro. Os poços serão novamente lavados com 50 µL de PBS por 15 minutos. Então o conteúdo sobrenadante dos poços será cuidadosamente removido para que os núcleos sejam corados com DAPI, uma coloração fluorescente que se liga ao DNA celular revelando o núcleo das células viáveis (ProLong Gold antifade reagent with DAPI, Invitrogen). Um grid com nove quadrados será confeccionado na tampa de uma placa de 96 poços e seis campos deste grid serão escolhidos aleatoriamente e utilizados para contar as células de cada um dos poços em microscópio óptico sob fluorescência. Serão contados os núcleos corados de

azul pelo DAPI (λ=350 nm) conferindo-se a localização com a marcação da actina F (λ=495 nm) para evitar a contagem de artefatos de imagem. O estudo será realizado em três seções independentes.

1.5.5.2. Crescimento celular em ambiente tridimensional

Scaffolds com as células do grupo controle e do grupo experimental serão injetados em placas de cultura de 35 mm de diâmetro com superfície inferior de vidro (P35G-0-10-C, MatTek Corp., Ashland, MA, EUA). Depois dos períodos de 1 e 7 dias as células serão fixadas e coradas como previamente descrito e observadas em microscópio confocal de varredura a laser (Zeiss LSM 510 META, Carl Zeiss) equipado com laser de argônio para FITC (λ=488 nm) e ultravioleta para DAPI (λ=350 nm) para captura de imagens em série Z. As imagens obtidas serão reconstituídas e projeções serão obtidas com uso de software específico (IMARIS 7.0, Bitplane Inc.).

1.5.5.3. Avaliação da morfologia celular por Microscopia Eletrônica de Varredura (MEV)

Scaffolds com as células, do grupo controle e do grupo experimental, serão injetados em placas de cultura de 35 mm de diâmetro com superfície inferior de vidro.As células serão fixadas em temperatura ambiente com 1% e 3% de gluteraldeído durante 1 e 24h respectivamente. Os scaffolds com as células semeadas serão desidratados utilizando etanol seriado (50%, 70%, 80%, 90%, 95%, e 100%) por 10 minutos cada.Os *scaffolds* serão mantidos *overnight,* cobertos com ouro/paládio, e logo após, submetidos à visualização por microscopia eletrônica de varredura.

1.5.5.4. Análise da produção de VEGF

As SHEDs serão cultivadas em uma placa de 24 poços em uma densidade de $1x10^5$ durante 24h. Então as células serão transfectadas com 2 ml de uma solução de α-MEM (Ad/rhVEGF$_{121}$/α-MEM)contendo adenovírus (em diferentes MOIs - 0, 5, 10, 20, 50 e 100) codificando o gene rhVEGF$_{121}$ durante 24h. O VEGF precisa ser exportado do ambiente celular para a região específica onde exercerá sua ação mitogênica. Assim, um peptídeo sinal será incorporado a esta molécula para confirmar a exportação de

VEGF e dessa forma comprovar seu potencial angiogênico sobre as células de origem endotelial.

Após o período de incubação as células serão lavadas três vezes com PBS e então suplementadas com meio fresco. Após os períodos de 3, 7 e 10 dias o meio será coletado e examinado para verificar o nível de VEGF liberado pelas células utilizando kit ELISA (R & D Systems).

1.5.5.5. Avaliação em co-cultivo da atividade mitogênica das SHED/rhVEGF$_{121}$

Para a avaliação da atividade mitogênica das células transfectadas com o gene de interesse serão utilizadas placas de co-cultivo de seis poços – Filtro com poros de 0.4-µm (Corning Life Sciences). As células alvo serão células endoteliais (HDMECs).As SHED/rhVEGF$_{121}$previamente transfectadas (com as cargas virais previamente citadas) serão tripsinizadas e então centrifugadas durante 10 minutos para que o pellet formado seja ressuspendido em meio de cultivo. Então as SHED/rhVEGF$_{121}$serão cultivadas em uma densidade de 1x10^5 células por poço da placa de co-cultivo (porção superior com o filtro - *insert layer*) contendo HDMECS na porção inferior (abaixo do filtro - *bottom layer*), também em uma densidade de 1x10^5. Em períodos de tempo pré-determinados – 0, 3, 7 e 10 dias – a parte contendo as SHED/rhVEGF$_{121}$será removida e então a proliferação das HDMECs será avaliada e comparada com a proliferação de células de mesma origem, cultivadas nos mesmos períodos de tempo sem estar em co-cultivo com as SHED/rhVEGF$_{121}$.

1.5.5.6. Viabilidade celular e diferenciação odontoblástica das células-tronco dentro do canal radicular de pré-molares humanos

Nesta parte do estudo serão utilizados pré-molares humanos com ápice aberto, indicados para extração por motivos ortodônticos. Os dentes serão obtidos de voluntários sadios jovens, os quais serão previamente informados sobre o conteúdo projeto e destinação do material biológico através de um termo de consentimento livre e esclarecido. Os dentes serão doados mediante a assinatura de uma autorização prévia do mesmo para o

seu uso imediato após a exodontia. Logo após a realização do procedimento cirúrgico de extração, os dentes serão acondicionados em MEM-α a 4 °C.

Para avaliar a viabilidade e o potencial de diferenciação odontoblástica células-tronco injetadas no canal radicular, os dentes terão suas coroas removidas com o uso de disco diamantado em baixa rotação realizando refrigeração com PBS dentro de uma capela de fluxo laminar.Então, o tecido pulpar será cuidadosamente removido com uso de limas compatíveis com o diâmetro do canal para evitar a instrumentação adicional do canal radicular. Os dentes serão imersos em soluções de álcool etílico com gradientes decrescentes (100, 90, 80 e 70° GL) realizando intensa agitação por 20 minutos cada, posicionados em placa de Petri com PBS e armazenados em estufa 37 °C por 14 horas**.** Para que as células injetadas nos condutos radiculares tenham acesso ao meio de cultura somente pelo ápice do dente, *inserts* para placa de 24 poços (Cell Culture Inserts 24 well 3µm pores, BD Falcon) receberão em suas membranas um pequeno corte realizado com bisturi #15c e serão inversamente posicionados em placas de cultura de células de 6 poços para que os dentes sejam transfixados na membrana. O conjunto será novamente posicionado na estufa por 2 horas para homogeneizar a temperatura da estrutura dental remanescente e em seguida o RADA-16 com SHED/20 ($6x10^6$ células/ml) será injetado nos condutos radiculares, contendo células controles ou transfectadas. Os controles negativos utilizados serão SHEDs em placa de cultura de 60 mm. Como controle positivo será utilizado mRNA isolado da pré-dentina (RNA odontoblástico) de terceiros molares extraídos de voluntários jovens e saudáveis (Conde et al., 2012).

A expressão gênica da diferenciação odontoblástica será avaliada a cada 7 dias coletando-se o conteúdo de cada grupo utilizando-se pipetas graduadas. A extração do RNA do material obtido será realizada com o uso de solução monofásica de fenol e guanidina isotiocianato (TRIzol Reagent, Invitrogen, Carlsbad, CA, EUA) seguida da síntese de cDNA (complementar DNA – cDNA) com uso de kit comercial (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen, Carlsbad, CA, EUA) e protocolo preconizado pelo fabricante. O RNA total extraído será utilizado em RT-PCR (Transcrição reversa – reação em cadeia de polimerase) para

amplificar os seguintes marcadores odontoblásticos: DSPP (*sense 5' gacccctttcattgacctcaact 3', antisense 5' tgccatttgctgtgatgttt 3'; 181 bp*), DMP-1 (*sense 5'caggagcacaggaaaaggag 3', antisense 5' ctggtggtatcttgggcact 3'; 213 bp*) e MEPE (sense 5' gcaaaagcacccatcgtatt 3', antisense 5' ctgccctctacaaggctgac 3'; 385 bp). Os primers específicos para cada um desses marcadores celulares serão desenvolvidos de acordo com a sequência de cDNA publicada no GenBank. Os produtos de RT-PCR serão analisados por eletroforese em gel de agarose a 1.5%, contendo SYBR Green, numa voltagem de 80KV durante 30 minutos. Desta forma, será procedida a densitometria óptica das bandas correspondentes aos marcadores celulares analisados, sendo para isso utilizado um software específico. As bandas serão normalizadas contra as densidades das bandas de GAPDH (glyceraldehyde-3-phosphate dehydrogenase), um gene constitutivo celular. Neste estudo RT-PCR será realizado em uma única etapa com auxílio do *Super Script one step RT-PCR com Platinum Taq Kit*, o que permite que a transcrição reversa e a amplificação dos genes estudados ocorram sequencialmente em um único tubo sob condições otimizadas.

1.5.6. Obtenção de polpa dental funcional *in vivo*

1.5.6.1. Preparo dos espécimes, implantação, recuperação e preparo para análises: SHEDs previamente marcadas com LacZ receberão a adição do vetor contendo VEGF com a carga viral considerada ideal nas avaliações *in vitro* acima descritas.

SHED(controles celulares positivos), SHED/LACZ+(SHED contendo LacZ), SHED/rhVEGF$_{121}$ (experimental)e SHED/rhVEGF$_{121}$/LACZ+ serão ressuspensas em RADA-16 (6x10^6 células/ml) e injetadas em raízes de pré-molares humanos com ápice aberto transfixados na membrana de inserts. Os controles negativos usados serão raízes onde os scaffolds serão injetados sem células. Os dentes serão armazenados em estufa 37°C por 30 minutos previamente à implantação (Tabela 1.). As raízes serão implantadas bilateralmente na região subcutânea do dorso de camundongos machos com imunodeficiência combinada grave (Severe Combined Immunodeficiency – SCID) com idades entre 5 e 7 semanas (CB.17 SCID, Charles River, Wilmington, MA, EUA). Os dentes serão randomicamente selecionados

(exceto os grupos com SHED/LACZ+) seguindo um critério que não permita que um camundongo receba dois dentes com um mesmo tipo celular.Os grupos contendo SHED/LACZ+ serão implantados em camundongos específicos para evitar que esses grupos sofram alguma influência da tetraciclina a ser injetada nos animais como será oportunamente descrito. As análises histológicas, morfológicas e imunoistoquímica serão realizadas com as células negativas para LacZ.

Tabela 1. Construção do Tecido Pulpar *In Vivo*

Obtenção de polpa dental funcional in vivo – Grupos Experimentais		
Grupo	Composição	n
Morfológico	Polpa de pré-molares (PM) com ápice aberto	1
PM+RADA16/20	Raiz de PM contendo somente o *scaffold* (Negativo)	4
PM+RADA16/20+SHED	Raiz de PM contendo *scaffold* e SHEDs (Positivo)	6
PM+RADA16/20+SHED+LACZ	Raiz de PM contendo *scaffold* e SHEDs LACZ+	2
Experimental		
PM+RADA16/20+rhSHED$_{165}$	Raiz de PM contendo *scaffold* e rhSHED$_{165}$	6
PM+RADA16/20+rhSHED$_{165}$+LACZ	Raiz de PM contendo *scaffold* e rhSHED$_{165}$LACZ+	2

Após a injeção intramuscular de solução 0.85 de quetamina 4,25 mg/kg (Ketaset, Fort Dodge, IA, EUA) e 15% de xilazina 0.25 mg/kg (AnaSed, Lloyd Laboratories, Sheanoah, IA, EUA) para promover a anestesia dos camundongos. O dorso dos animais receberá uma incisão superficial e em ambos os lados a pele será divulsionada da camada muscular com uso de tesoura sem ponta. Os dentes serão inseridos nas lojas cirúrgicas criadas, a incisão reduzida e unida com uso de cola veterinária (3M Vetbon Tissue Adhesive, 3M, Saint Paul, MN, EUA). Para evidenciar a que o possível tecido conjuntivo neoformados é capaz de produzir dentina, os animais receberão injeções de tetraciclina hidroclorada (T3383, Sigma-Aldrich Corp. St. Louis, MO, EUA). No décimo dia após a implantação, os camundongos de cada grupo (excetuando-se os LACZ+) receberão uma injeção intraperitoneal de tetraciclina (41,6 nmol/g de massa corporal) a qual será administrada mais 3 vezes a cada cinco dias. Depois da última administração, seguir-se-á um período de 10 dias sem injeções.

Após 35 dias da implantação, todos os camundongos serão submetidos à eutanásia por meio de dosagem excessiva de solução anestésica seguido de deslocamento cervical. Logo após os implantes serão recuperados. As raízes utilizadas para verificar a formação do tecido pulpar

serão imediatamente armazenadas em solução de formalina tamponada 10% por 24 horas a 4 °C para promover a fixação dos tecidos obtidos e descalcificados com uso de solução de ácido hidroclorídrico e EDTA (Decalcifier II, Surgipath Medical Industries, Inc., Richmond, IL, EUA). A descalcificação será realizada de 8 a 22 horas em temperatura ambiente. A partir da quarta hora do inicio da descalcificação os dentes serão retirados da solução para averiguar a sua rigidez e a necessidade de continuar com o processo de desmineralização. A partir dos espécimes serão confeccionadas lâminas histológicas coradas com eosina e hematoxilina (H&E) e lâminas sem coloração serão utilizadas para realização das análises imunoistoquímicas.

Os espécimes utilizados para verificar a neoformação dentinária serão armazenados em PBS imediatamente depós a explantação e seccionados em seus longos eixos com uso de disco diamantado em baixa rotação e intensa refrigeração e examinados empregando microscopia confocal (Olympus Fluoview 500 Laser Scanning Microscope, Olympus, Melville, NY, EUA) utilizando laser ultravioleta (405 nm, 35 mW) filtro de excitação (405–488 nm) e filtro de barreira (465–495 nm). Os controles negativos utilizados Serão espécimes contendo SHED e $rhSHED_{121}$ injetadas com RADA-16 implantados em camundongos que não receberam injeções de tetraciclina e como controle positivo será utilizado o incisivo central de um camundongo.

1,5.6.2 Análise das características morfológicas

O número de células que compõem a “camada odontoblástica” da polpa neoformada será determinado contando-se o numero de células localizadas na parede dentinária. Para tanto, 10 campos randomicamente selecionados de três lâminas obtidas com cada linhagem celular receberão um grid contendo 5 retângulos. As células localizadas no interior de cada retângulo serão demarcadas com uso de software específico (Adobe Photoshop 7.0, Adobe, Seattle, WA, EUA) e esta imagem será avaliada com uso de software para análise de imagens (ImageJ, NIH, Bethesda, MD, USA) o qual fornecerá o número de odontoblastos presentes em 125 μm de parede de dentina por campo analisado. A determinação da densidade

microvascular será realizada contando-se os vasos sanguíneos de 10 campos aleatoriamente (40x) selecionados em 3 lâminas por grupo pelo autor e também por um avaliador “cego” para este estudo. O controle positivo utilizado será um pré-molar humano com ápice aberto.

1.5.6.3 Análise imunoistoquímica

Lâminas com cortes histológicos serão desparafinizados para a recuperação de epítopos, a qual será realizada com as lâminas imersas em solução de recuperação de antígenos 1x (Target Retrieval Solution 10x, Dako, Carpinteria, CA, EUA) por 20 minutos a 120 °C seguido de 20 minutos a 95 °C. As lâminas serão lavadas em solução tampão 1x (Dako Wash Buffer 10X, Dako, Carpinteria, CA, EUA). O bloqueio da peroxidase endógena será realizado com peróxido de hidrogênio 3% por 5 minutos (Peroxidase 1, Dako Cytomation EnVision+HRP (AEC), DakoCytomation, Inc. Carpinteria, CA, EUA) seguido do bloqueio de coloração não específica efetuado por 15 minutos após a realização de cada uma das etapas mencionadas. Nos diferentes protocolos de imunoistoquímica serão realizadas duas lavagens por 10 minutos realizando suave agitação com solução tampão também (Background Sniper, Biocare Medical, Walnut Creek, CA, EUA) ambos em temperatura ambiente. A marcação para Fator VIII será realizada com uso de anticorpo anti-Factor VIII (Factor VIII Related antigen Ab1, Neomarkers, Fremont, CA, EUA). O anticorpo utilizado na concentração 1:400 (Dako Antibody Diluent, Dako, Carpinteria, CA, USA) será incubado por 16 horas a 4°C. A incubação do polímero (Labelled Polymer-HRP Anti-Rabbit, DAKO Cytomation EnVision+HRP (AEC), DakoCytomation, Inc. Carpinteria, CA, EUA) será realizada por 40 minutos em temperatura ambiente e a reação será revelada sob observação em microscópio óptico utilizando cromógeno 3-amino-9-etil-carbazol (3-amino-9-ethyl-carbazole – AEC, AEC chromogen substrate buffer (DAKO Cytomation EnVision+HRP (AEC), DakoCytomation, Inc. Carpinteria, CA, EUA) por aproximadamente 25 minutos. A identificação de antígeno nuclear de proliferação celular (Proliferating Cell Nuclear Antigen – PCNA) será realizada utilizando anticorpo (PCNA (F-2), Santa Cruz Biotechnology, Inc. Santa Cruz, CA, EUA) em concentração de 1:300 por 16 horas a 4 °C. Os

cortes serão incubados com a sonda e polímero contra anticorpo criado em coelhos (MACH3 Mouse/Rabbit-Probe HRP Polymer Kit, Biocare Medical, Walnut Creek, CA, EUA) por 40 minutos cada em temperatura ambiente. A reação será revelada com solução de 3-3'-diamonibenzidina (3-3' diaminobenzedine – DAB, DAB Chromogen Kits, Biocare Medical, Walnut Creek, CA, EUA) sob observação em microscópio óptico por aproximadamente 45 segundos. As células positivas para PCNA serão contadas em 10 campos escolhidos aleatoriamente em 3 lâminas por grupo pelo autor e um avaliador cego para este trabalho utilizando softwares de processamento e analisador de imagens.

A revelação em microscopia óptica será realizada por aproximadamente 3 minutos. O controle negativo para LACZ será feito com pré-molar humano extraído. Os resultados das imunoistoquímicas das polpas formadas com SHED e scaffolds injetáveis serão comparados com colorações obtidas em pré-molar com ápice aberto extraído devido à necessidade ortodôntica (Controle Positivo). O controle negativo de todas as reações imunoistoquímicas será realizado com anticorpo contra imunoglobulina G (IgG controle) e a montagem das lâminas será feita com solução aquosa (VectaMount AQ, Vector Laboratories Inc., Burlingame, CA, EUA).

1.6. Cronograma e plano de atividades

Ano 2012

Meses de Julho/Agosto/

- Levantamento Bibliográfico do assunto
- Treinamento para o desempenho das técnicas de transfecção gênica em células-tronco de origem pulpar.
- Levantamento da documentação necessária exigida pela CAPES para realização de estágio de Doutorado no exterior (PDSE).
- Levantamento da documentação para obtenção do visto de imigração J-1 junto a Universidade de Michigan
- Treinamento de bolsistas de Iniciação Científica

Mês de Outubro

- Levantamento Bibliográfico do assunto
- Continuação do treinamento para o desempenho das atividades laboratoriais
- Execução de testes piloto para realização da transfecção e avaliação da proliferação de células-tronco da polpa dental de dentes decíduos no NCT-Bio - UFPel
- Treinamento de bolsistas de Iniciação Científica
- Organização Final da documentação necessária para o estágio PDSE

Meses de Novembro/Dezembro

- Inicio do estágio PDSE na Escola de Odontologia da Universidade de Michigan sob orientação do Professor Jacques Nör
- Treinamento no Laboratório da Universidade de Michigan

Ano 2013

Meses de Janeiro/ Fevereiro/Março

- Levantamento Bibliográfico do assunto.
- Preparo dos Scaffolds Injetáveis (RADA-16), Cultivo Inicial das SHED para realização dos ensaios anteriormente propostos

- Transfecção adenoviral das SHED (rhVEGF$_{121}$) utilizando o sistema de co-transfecção HEK293 como sistema celular de hiperprodução em diferentes MOIs (0, 5, 10, 20, 50 e 100).
- Avaliação da taxa ótima, em MOI, para transfecção das SHED.
- Semeadura das células nos *scaffolds* injetáveis para inicio da avaliação das propriedades de diferenciação, proliferação e potencial mitogênico das células transfectadas com relação às células não alteradas.
- Avaliação da morfologia celular por Microscopia Eletrônica de Varredura (MEV)

Meses de Abril/Maio/Junho

- Levantamento Bibliográfico do assunto.
- Análise dos resultados obtidos nos ensaios preliminares realizados no primeiro trimestre para que os testes do segundo trimestre sejam realizados com a utilização de concentrações ótimas de reagentes e carga viral transfectadas às células
- Coleta de dentes na Clínica CBMF da Escola de Odontologia da Universidade de Michigan.
- Armazenamento dos dentes para realização dos ensaios *In Vivo*.
- Avaliação prévia em placas de cultivo da viabilidade, liberação de VEGF e diferenciação odontoblástica das células *in vitro.*
- Avaliação da atividade mitogênica das SHED transfectadas sobre células endoteliais
- Avaliação da formação de tecido pulpar *in vitro* em canais de dentes humanos

Meses de Julho/Agosto/Setembro

- Levantamento Bibliográfico do assunto.
- Análise dos resultados obtidos nos ensaios preliminares realizados no primeiro semestre para dar início aos ensaios *in vivo*.
- Inicio dos ensaios *In Vivo* realizando a preparo dos espécimes (Dentes coletados no segundo trimestre de 2013), implantação, recuperação e preparo para análises.

Meses de Outubro/Novembro/Dezembro

- Inicio da Redação dos artigos científicos produzidos durante o estágio PDSE para submissão em Periódicos Qualis A1;
- Análise histológica e imunoistoquímica dos tecidos produzidos *In Vivo*.
- Análise final dos resultados produzidos durante o estágio PDSE.
- Retorno do PDSE (Outubro)
- Redação da Tese

Ano de 2014

Meses de Janeiro/Fevereiro/Março

- Submissão dos artigos científicos produzidos durante o estágio PDSE para submissão em Periódicos Qualis A1
- Redação da Tese

Meses de Abril/Maio

- Defesa da Tese
- Entrega da Versão Final da Tese de Doutorado

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2. Relatório do trabalho de campo (Alteração de projeto)

Nesta seção estão relatadas as mudanças de trajetória no rumo da pesquisa previamente proposta no projeto de qualificação realizado em junho de 2012. Originalmente foi proposta a execução de um projeto (Descrito no item 1) junto à universidade de Michigan (MI – USA), no laboratório de Angiogênese coordenado pelo professor Jacques Eduardo Nör. O projeto original foi devidamente qualificado e aprovado pela banca examinadora. Também o plano de estudos referente ao projeto de pesquisa foi analisado e aprovado pelo Prof. Nör. A chegada do proponente aos Estados Unidos, para o inicio das atividades, estava prevista para novembro de 2012. Entretanto, no período entre a qualificação e o inicio do doutorado sanduíche, o professor Flávio Fernando Demarco foi contemplado com duas bolsas de Pós-Doutorado DOCFIX, programa de fixação de recém-doutores de longa duração, para atuarem em projetos de pesquisa e desenvolvimento científico, tecnológico e de inovação em núcleos de inovação e transferência de tecnologia (Neto) e nos programas de pós-graduação (PPG) gaúchos.

O Programa de Pós-Graduação em Odontologia (PPGO) da UFPel apresenta cinco linhas de pesquisa dentro das áreas de concentração em Dentística e Clínica Infantil. O presente projeto é de extrema importância para reforçar a consolidação especialmente de duas linhas: (i) Desenvolvimento, caracterização e controle de biomateriais, nanotecnologia e suas aplicações na odontologia, e (ii) Biopatologia, biologia molecular, engenharia tecidual, cultivo celular e biocompatibilidade dos materiais dentários. Dessas duas linhas de pesquisa desenvolvem-se aproximadamente 70% da produção científica do Programa. Com a agregação de dois novos recém-doutores pelo programa DOCFIX, será possível redimensionar a ampliação do PPGO, que será focado no nível de doutorado em Biomateriais ou Materiais Dentários para o desenvolvimento de materiais e tecnologias para o mercado nacional, uma vez que nosso programa é um dos únicos no país que trabalha com a parte de desenvolvimento de produtos odontológicos, com patentes registradas, produtos em vias de lançamento e parceria com empresas do ramo odontológico. Também, a aquisição de dois pós-doutores é de extrema importância

para a consolidação da linha de pesquisa em engenharia tecidual de tecidos dentais, na qual o PPGO se apresenta como um dos pioneiros no país desde o ano de 2008 quando estabeleceu uma parceria com a UMICH durante o pós-doutorado do Prof. Demarco.

Durante os 30 meses como bolsista (CAPES) de doutorado, o candidato teve como foco de seus estudos a área de engenharia tecidual e biologia molecular, apresentando uma produção científica consistente com artigos internacionais de qualis entre A1 – B1, além de ampla participação em congressos nacionais e internacionais. Durante o período de doutoramento o candidato produziu dois artigos (descritos a seguir) que se encontram dentro do escopo do projeto original e desta forma cumprem os requisitos para serem utilizados no processo público de defesa.

O caráter de urgência da defesa foi relatado ao colegiado do PPGO e prontamente acatado por seus membros, dada a importância da manutenção das bolsas de pós-doutorado obtidas via edital (EDITAL FAPERGS/CAPES 09/2012).

3. Introdução

Atualmente 85% da população mundial apresenta necessidade de reparos, em diferentes magnitudes, nas estruturas da região craniofacial (SCHELLER et al., 2012). Dentre estas desordens a perda dental compreende uma porção significativa e afeta pessoas de diferentes faixas etárias (POLZER et al., 2010). As principais causas de perda dental são a doença periodontal (GAMONAL et al., 1998), o trauma dental (ALANI et al., 2012) e a doença cárie.

Na população brasileira a necessidade de prótese dental é identificada já aos 15 anos (SB Brasil 2012) sendo que aos 24 anos de idade 29,7% da população já apresenta tal necessidade (CORREA et al., 2010). Neste contexto, dados alarmantes são detectados na população adulta (35 - 44 anos), da qual 68,8% necessitam da reposição de um ou mais dentes (SB Brasil 2010). Apesar de o edentulismo não ser uma condição que represente risco de morte, exerce um impacto crucial na qualidade de vida dos indivíduos afetados. Além do prejuízo na função mastigatória, a perda dental proporciona problemas de ordem nutricional, afeta a fonação e a estética (ROUMANAS, 2009), e proporciona limitações sociais (POLZER et al., 2010). Os materiais sintéticos, restauradores e protéticos utilizados atualmente, apesar de serem de fácil aplicação e possuírem relativo baixo custo, não são capazes de induzir a regeneração da parte afetada- ossos maxilares, dentes, vasos sanguíneos do organismo receptor (FERRACANE et al., 2010). Assim, estes materiais proporcionam um reparo limitado da função e estética do tecido original (SMITH et al., 2008).

Uma teoria revolucionária trouxe à tona a possibilidade da regeneração completa de tecidos e até mesmo órgãos; a Engenharia Tecidual foi proposta como um campo interdisciplinar que funde princípios e inovações da Engenharia e das Ciências Biológicas (LANGER; VACANTI, 1993). Este campo da ciência tem por objetivo o reparo ou substituição de tecidos e órgãos baseado em três pilares fundamentais: as moléculas bioativas - Fatores de crescimento (FC), moléculas de origem proteica, com capacidade de se ligarem a receptores específicos presentes na membrana celular, e a partir daí reger o comportamento dessas estruturas

(MATTUELLA et al., 2007); *os scaffolds* – estruturas tridimensionais que servem como substrato para a adesão e proliferação das células simulando assim a matriz extracelular; e as células-tronco que possuem alta capacidade clonogênica, de proliferação e capacidade de diferenciação em uma ampla gama de tecidos (LANGER; VACANTI, 1993). Na Odontologia, o desenvolvimento de terapias que visam à regeneração ou reparo biológico das estruturas dentais tem produzido um crescente interesse entre os pesquisadores da área (SMITH et al., 2008).

A polpa dental é um tecido conjuntivo de características únicas que possui como função primordial a formação de dentina (BJORNDAL; MJOR, 2001) a partir de uma matriz orgânica secretada pelos odontoblastos (LINDE; GOLDBERG, 1993). Devido à natureza pós-mitótica das células odontoblásticas, a polpa possui uma capacidade de regeneração limitada (LINDE; GOLDBERG, 1993). Todavia, este tecido possui um requintado mecanismo de defesa que permite o reparo das estruturas do complexo dentino-pulpar – CDP (GOLDBERG; SMITH, 2004). Neste contexto, lesões crônicas desencadeiam eventos moleculares que sinalizam para os odontoblastos intensificarem sua atividade secretória e assim, produzir dentina reacional no local subjacente à injúria (GOLDBERG; SMITH, 2004).

Sólidas evidências (GRONTHOS et al., 2002; GRONTHOS et al., 2000; MIURA et al., 2003) demonstram que a polpa, de dentes permanentes e decíduos, possui em seu interior uma população de células mesenquimais progenitoras denominadas “Dental Pulp Stem Cells (DPSC)” e “Stem Cells from Exfoliated Human Teeth (SHED)”, respectivamente. Quando o CDP é submetido a injúrias de rápida progressão, como lesões de cárie aguda ou trauma dental que proporcionam a destruição dos odontoblastos (BJORNDAL; MJOR, 2001) há uma sinalização para que esta população de células progenitoras migre para o sitio da injúria (TECLES et al., 2005). Após o processo de migração essas células se diferenciam em odontoblasto símiles e iniciam a secreção de dentina reparadora (GOLDBERG; SMITH, 2004). Entretanto, o processo de formação de dentina reparadora não se dá de forma espontânea; é necessária a aplicação de materiais sintéticos que estimulem este evento biológico (BJORNDAL; MJOR, 2001).

Tradicionalmente o material utilizado para induzir o reparo do CDP é o Hidróxido de Cálcio – Ca(OH)2 – e mais recentemente o Agregado de Trióxido Mineral – MTA (FORD et al., 1996). Estes produtos induzem a formação de tecido mineralizado no local da exposição pulpar, e devido ao seu elevado pH possuem

propriedades bactericidas e bacteriostáticas. Apesar dos resultados satisfatórios esses materiais não possuem um alvo de ação específico (ALMUSHAYT et al., 2006). Quando em contato direto com a polpa provocam uma necrose superficial que proporciona comprometimento de sua estrutura; o reparo se dá à custa de tecido sadio (HILTON, 2009). Além disso, as terapias vitais da polpa possuem indicações restritas e geralmente seu prognóstico em longo prazo não é promissor (TUNA, 2009). Desta forma, a terapia endodôntica convencional é indicada em situações de envolvimento pulpar, justamente por possuir um prognóstico mais favorável (DEMARCO et al., 2005). Contudo, a excisão total do tecido pulpar apresenta desvantagens consideráveis no que concerne à manutenção da estrutura dental na arcada dentária. O tratamento endodôntico causa uma perda considerável de tecido dentinário tendo como consequência um dente não vital e mais suscetível a fraturas (DEMARCO et al., 2005). Isto é especialmente crítico quando se trata de trauma dental em dentes permanentes de pacientes jovens (NOR, 2006)

A viabilidade e manutenção do órgão dental na arcada dentária estão diretamente relacionadas com a vitalidade do tecido pulpar, uma vez que a polpa vital proporciona nutrição e exerce a função de receber e perceber estímulos patogênicos (BJORNDAL; MJOR, 2001). Além disso, a manutenção da vitalidade pulpar é essencial para a contínua deposição de dentina (BJORNDAL; MJOR, 2001). A interrupção da dentinogênese, em dentes permanentes jovens, dá origem a características morfológicas que podem complicar a realização de possíveis tratamentos endodônticos, como câmara pulpar volumosa, menor espessura de dentina radicular e formação incompleta da estrutura apical (NOR, 2006). Assim, empregar a engenharia de tecidos para a obtenção de tecido pulpar que permita a completa formação dos tecidos e anexos radiculares é uma estratégia que se mostra cada vez mais atrativa e factível.

O *scaffold* proporciona substrato em 3 dimensões para desenvolvimento das células, atuando como matriz para a regeneração tecidual (DEMARCO et al., 2011), podendo ainda ser empregado como sistema de carreamento para direcionar respostas celulares específicas (GALLER et al., 2012). A seleção do material e técnica para a confecção de um *scaffold* é de extrema importância no desenvolvimento de terapias regenerativas da polpa dental. Atualmente os polímeros sintéticos - PLLA, PGA, PLGA -, polímeros naturais - Colágeno Tipo I -, a base de cálcio HÁ/TCP-, estão entre os materiais mais utilizados para a confecção

de scaffolds (ZHANG, H. et al., 2012). Além desses surgem como alternativas o emprego matriz extracelular dentinária (ZHANG, H. et al., 2012), Fibras de Seda (ZHANG, W. et al., 2011) e principalmente os Polímeros Anfifílicos (PA) Auto-Organizáveis (GALLER et al., 2008; GALLER et al., 2011; GALLER et al., 2012). Devido a esta ampla gama é difícil estabelecer um material para ser utilizado como padrão na engenharia tecidual do tecido pulpar. Apesar dos polímeros sintéticos serem biocompatíveis, baratos e facilmente manipuláveis (características como velocidade de degradação e resistência das fibras produzidas são facilmente controladas), o que permite sua reprodutibilidade em larga escala, eles não reproduzem adequadamente as atividades biológicas da MEC (GALLER et al., 2012). O colágeno por sua vez, possui todas as moléculas bioativas presentes na MEC, entretanto é extremamente difícil manipular sua estrutura física e composição (YANG et al., 2012). Além disso, pode suscitar resposta imune ou conter impurezas patológicas. Muitos estudos estão sendo desenvolvidos para melhorar a funcionalidade dos *scaffolds* e suas futuras aplicações (GALLER et al., 2008; GALLER et al., 2011; GALLER et al., 2012; ZHANG, H. et al., 2012; ZHANG, W. et al., 2011).

Além da composição química do material, a interface tecido-*scaffold* é fortemente influenciada pelas propriedades físicas do scaffold. A estrutura, forma geométrica, espessura das fibras e tamanho dos poros de um material utilizado com *scaffold*, afetam a capacidade de proliferação, adesão e diferenciação celular (SANTANA et al., 2012). Para que possam mimetizar a MEC, estas estruturas devem possuir um tamanho médio de poros que permita o crescimento, migração e nutrição das células nele implantadas. Quando o tamanho dos poros é muito reduzido a capacidade de migração das células é comprometida, o que pode limitar a difusão de nutrientes e a remoção de metabólitos (DEMARCO et al., 2011). Por outro lado, quanto maior o tamanho dos poros, o trânsito de nutrientes e a eliminação de metabólitos são facilitados. Entretanto, esta característica irá influenciar de forma negativa a adesão das células tronco, além de comprometer propriedades mecânicas do material (LAWRENCE; MADIHALLY, 2008).

Na engenharia tecidual do tecido pulpar, diferente da engenharia tecidual de osso (MURPHY; HAUGH; O'BRIEN, 2010; O'BRIEN et al., 2005), não existe uma padronização das características físicas dos scaffolds que favoreçam a regeneração da polpa dentro de um ambiente inóspito como o canal radicular.

3.1 Objetivo

- A fim de identificar as estratégias mais promissoras, baseadas na utilização de células-tronco adultas, para produção de tecido pulpar funcional, bem como as principais estratégias utilizadas para aprimorar a interface dinâmica existente entre estas células e os *scaffolds,* o nosso primeiro objetivo foi realizar uma revisão sistemática da literatura.

- Objetivou-se, também, realizar um estudo *in vitro*, aplicando o modelo do *Tooth Slice/Scaffold* (TS/S) (GONÇALVES et al., 2007) para avaliar a influência de dois tamanhos de porógenos na capacidade de proliferação e diferenciação odontoblástica de DPSC (GRONTHOS et al., 2000).

3.2 Hipótese

No estudo experimental trabalhamos com a hipótese de que os scaffolds a base PLLA com porosidade de 150-250μm seriam adequados para a proliferação e diferenciação odontoblásticas, assim como os scaffolds com porosidade de 251-450μm, os quais já tiveram sua eficiência comprovada em estudos prévios (CORDEIRO et al., 2008; DEMARCO et al., 2010; CASAGRANDE et al., 2010)

4. Artigo 1 : Stem Cell-Based Pulp Tissue Engineering: Variables enrolled in translation from the bench to the bedside, a systematic review of literature. Formatado segundo as normas do periódico Journal of Biomedical Materials Research Part A (Qualis: A1– JCR: 2.147)

Stem Cell-Based Pulp Tissue Engineering: Variables enrolled in translation from the bench to the bedside, a systematic review of literature.

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Abstract

Recent studies have been showing that, in the future, dental pulp would be regenerated using Stem Cell-Based Therapies (SC-BT). Such studies show that the interaction between stem cells and the scaffolds plays a critical role in the generation of a “friendly cell” microenvironment. This study’s aim was to review systematically the literature searching for techniques used to regenerate the pulp tissue using SC-BT and to review the tools used to improve the interaction between stem cells and the scaffolds. Specific inclusion and exclusion criteria were predetermined. Electronic search was carried out on the SciVerse Scopus (SS), ISI Web Science (IWS) and Entrez PubMed (EP) using selected keywords. The search yielded 77 papers, out of which 31 full-text papers were included by three independent reviewers to data extraction, which was done pooling the following specific criteria: Influence of physicochemical characteristics of scaffolds over the cell behavior; Influence of cue environment on Stem cell differentiation towards odontoblast-like cells and Pulp-like tissue formation; VEGF expression and Angiogenesis. The findings collected here show that the pulp regeneration is not anymore an achievable goal. The studies included here shows exciting results indicating that the transition from the bench to the bedside is close.

Introduction

The identification of a highly proliferative and multi-lineage subpopulation of cells[1,2] capable of differentiating towards odontoblast phenotype had brought together the possibility to apply tissue engineering (TE) principles to regenerate the dental pulp[3]. Tissue engineering is an interdisciplinary field applying the principles of engineering and life sciences for the development of biological struts to restore, maintain, or improve body's tissues function[4]. The essential constituent of TE are scaffolds, stem cells, and signaling molecules, which form "the triad" essential for the Stem Cell-Based Therapies – SC-BT (for details review Demarco et al.,[3]). Thenceforth, the development of a new dental pulp tissue applying SC-BT has produced a crescent interest. The human odontoblasts represent a terminally specialized pulp cell population unable to regenerate or duplicate itself, which limit the regenerative ability of dental pulp[5]. Thus, infection, exposure, trauma, and chemicals frequently lead to the dental pulp necrosis, which is typically treated with inert materials to substitute the pulp tissue, leaving as life-lasting sequel, a non-vital and weakened tooth[6].

Recent studies[7-10] have proven that dental pulp can be regenerated using SC-BT when implanted into live organisms. Taking everything into account, the interaction between the stem cells and the materials applied as scaffolds plays a critical role in the generation of a "cell-friendly" microenvironment, which must be conducive to the regeneration of dental structures. The ECM is an intricate network of nanofibrous proteins providing anchorage and biological guide to regulate the cells behavior[11]. In TE the scaffold's role is to act as an ECM biomimetic, on account of that it should be able to retain bioactive molecules into its structure to orchestrate the cell proliferation, migration and differentiation like the natural ECM. If one takes into account the challenges imposed by the quest for engineering a

connective tissue confined into the human dental root and the environment-imposed need for recruiting blood vessels and neuronal structures solely through the apical foramina the role of the scaffold's interaction with stem cells and the environment becomes even more critical. Thus, equally as important as the stem cell behavior is the scaffold interaction with these cells in the TE paradigm.

The aim of this study was to carry out a systematic review of literature to identify the most promising stem cell-based therapies techniques used to produce a functional pulp tissue. Besides, we looked up the aim strategies used by researches to improve the interface between the scaffolds and stem cells.

Materials and Methods

Our systematic search was carried out into the SciVerse Scopus®, ISI Web of Science® and Entrez PubMed up to July 2012. Detailed search strategies were developed for each database searched. In general, to perform the search, we have used the string of following specific keywords and Boolean operator: “Dental Pulp”, AND “Tissue Engineering” AND Scaffold AND “Stem Cells”. At the SciVerse Scopus, each one of the Keywords was included in the specific field “Article Title, Abstract, and Keywords”. In the Web of Science the keywords were placed into specific field “Topic”, present in the database layout, which conducts the search on Title, Abstract, Author Keywords and Keywords Plus. At the Entrez PubMed we have used the “advanced search”, in order to our investigation in was similar to Scopus and Web of Science.

Inclusion criteria used: a) studies should be original research articles targeting to regenerate de novo pulp/dentin-like tissue applying SC-BT; b) studies evaluating stem cells’ attachment, proliferation and/or differentiation towards odontoblastic phenotype, when seeded onto different types of scaffolds;

Exclusion criteria used: a) studies using dental stem cells aiming to regenerate or reconstruct other tissue rather than pulp-like tissue; b) studies using any kind of therapy rather than SC-BT; c) literature reviews were excluded; d) Studies using the blood clot technique e) studies wrote in another language than English.

Studies selection: two independent reviewers (MCMC, FFD) read the titles and abstracts of all reports identified throughout the electronic searches. For studies appearing to meet the inclusion criteria, or for which there were insufficient data in the title and abstract to make a clear decision, the full report was obtained. The full reports obtained from the electronic search were assessed independently by the same reviewers to establish

whether the studies met the inclusion criteria or not. Any disagreement was solved by discussion in order to achieve consensus. All studies meeting the inclusion criteria underwent a validity assessment and data extraction.

Data Extraction and Analysis: After the data analysis, the data extraction was done using the follow specific issues:

i. Influence of scaffold's chemical properties over the cell behavior;

ii. Influence of physical characteristics of scaffolds over the cell behavior;

iii. Strategy applied to improve the Stem cell/Scaffold interface

iv. Influence of cue microenvironment on stem cell differentiation towards odontoblast-like cells and Pulp-like tissue formation

Results

Studies Selection: An initial search at SciVerse Scopus resulted in 77 articles, 28 of which complied with the inclusion criteria and were selected for review (Table 2). At the Web of Science 62 articles were found and 4 additional relevant studies[12-15] that were not found at SciVerse Scopus were incorporate to our review. At the PubMed 41 records were obtained but no further articles were identified as suitable for our study. In the search carried out by us not necessarily the same records were obtained at the different database used. For this reason, 32 full text manuscripts were selected for inclusion in our systematic review and among those, only 8 records were common to the three databases (Table 2). Between the included 32 full text papers, four[14-17] were excluded because they did not meet the inclusion criteria. After that, 28 articles (Table 3) were analyzed for data extraction.

Influence of chemical characteristics of scaffolds over the cell behavior: Proof-of-principle studies selected (Table 3) provided information about the use of a wide range of synthetic and natural-based materials as scaffolds to support the adhesion and proliferation of stem cells. The polymeric (PLLA, PLGA, PLLA/HA), natural (Collagen I) and Calcium-based (HA/TCP) scaffolds were the most applied materials to construct the porous scaffolds. D,D-L,L-polylactic acid (OPLA) and collagen scaffolds were shown to be efficient materials to support stem cells attachment, proliferation and differentiation in vivo [9,10,18-20]. Moreover, it was possible to observe the potential of new materials called Peptide-Amphiphile – PA – [7,8,21], which are able to mimic better the ECM characteristics due to its ability to form nanofibrous under physiologic pH and temperature.

Influence of physical characteristics of scaffolds over the cell behavior: The physical characteristics of scaffolds were poorly explored as variable in the studies found. The

physical properties evaluated and related to de novo pulp regeneration were scaffolds' pore size and geometry [22-26]; fiber diameter[7,8,21] and material volume[25].

Strategies applied to improve the Stem cell/Scaffold interface: into the selected studies, the aim strategy used was the incorporation of bimolecular (TGF-β1, BMP-2 and DMP1) into scaffold structures[7,8,20,27] or culture medium[28,29]. With regard to the physical properties, we observed that the attempts to control the fiber width to produce nanofibrous polymers like the Self-Assembly Peptides[7,8,21] was a successful strategy.

Odontoblastic differentiation and pulp-like tissue formation: All studies included depicted DPC differentiation towards odontoblastic phenotype or *de novo* pulp-like tissue regeneration. It was interesting to note that the relationship between the scaffolds and the environment is critical for the novo pulp tissue regeneration. Studies using TS/S showed relevant results demonstrating the role of growth factors fossilized into dentin matrix for stem cells differentiation, which were able to regenerate pulp tissue[10,18,19,22]. Studies[9,13,25,30] have provided evidence about the influence of environment over DSC differentiation. The pre-conditioning of dentin walls with Ethylenediamine tetra acetic acid (EDTA) seemed to be imperative for success (Table 4). Furthermore, the application of SC-BT allows the regeneration of pulp tissue with blood vessel even disseminated into the tissue[7-10,19,23]. Such ability was attributed to the VEGF from dentin matrix and inherent ability of stem cells to expresses pro-angiogênico molecules[10].

Discussion

The first studies included in our review[12,31] showed crucial evidences to provide the basis to realize the development of dental tissues using the TE principles. All of subsequent studies have shown that an adequate interface can be created between the dental stem cells and the materials used as scaffolds (Table 3). Still, some studies evaluating the influence of scaffold's physical characteristics over the DSC behavior had shown some controversial results. Zhang et al[32] have demonstrated, in vitro, the ability of rat stem cells to attach onto HA/TCP scaffolds. Huang et al[13] demonstrated that collagen matrix may not be a suitable scaffold for pulp tissue regeneration due to its significant contraction. On the other hand, Gebhardt et al[33] have shown that open-cell OPLA and collagen were more suitable matrix for human DPSC survival, whereas calcium phosphate bio ceramic had the highest occurrence of cell apoptosis. It is interesting to note that neither of the above mentioned studies had used any additional growth factor (GF) other than those existing into supplemented culture medium.

GFs have a distinct role in formation of highly organized and well-structured tissues, like dental pulp[34]. Gotileb et al[29] have used TGF-β1 and/or BMP-2 trying to improve the SHED attachment and proliferation into polymeric scaffolds. However, no obvious difference was observed regarding the cell activity into constructs treated with GFs. Probably, such negative result was observed due the GF incorporation had been done by dripping diluted molecules over the scaffold structure, rather than bind the biomolecules into the scaffold structure. The authors suggested that TGF-β1 and BMP-2 were leached out by the culture medium before they could be incorporated by the cells. Such evidence was fundamental in developing strategies aiming to improve the interface between the stem cells and the scaffolds.

Yang et al[27] showed that BMP-7 gene-activated chitosan/collagen scaffolds were better for cell viability and proliferation than regular scaffolds and promoted odontogenic differentiation. In addition to the GFs, non-collagenous proteins (NCP) found in dentin matrix have been used to improve Dental Stem Cells (DSC) proliferation and odontoblastic differentiation. The addition of Dentin Matrix Protein 1 (DMP1) in Collagen scaffolds allow the differentiation of DPSCs towards dentin matrix-synthesizing cells, and improves the tissue formation into a tooth slice implanted in vivo[20]. Yet, another studies obtained success applying growth factors like VEGF, FGF2 and BMP-2 into the scaffold's structure[8]. Despite the success observed in studies using natural scaffolds, a substantial number of papers established that the polymeric scaffolds were able to support the DSC differentiation and pulp-like tissue formation into empty pulp chamber [9,10,18,19,22,29]. It is clear that, even though in assays carried out in vivo, there was not a consensus about the ideal material to construct the scaffolds that would better mimic the pulp ECM. Yet, the fusion of biomolecules, like GFs and NCPs, into the scaffolds structure has been considered a putative strategy to improve the interface between scaffolds and DSCs.

To simulate the ECM, characteristics other than the chemical properties of the scaffolds must be optimized. The pores distribution, pore size and fiber thickness also influence the cell behavior, too. Here, we could observe few studies evaluating, or trying to improve, the physical characteristics of scaffolds[22,23,25]. PLLA nanofibrous scaffolds fabricated by an electrospinning process were believed to be a potential cell carrier in Dentin Tissue Engineering (DTE). Actually, nanofibers have improved the stem cell behavior since this kind of structure simulates better the spatial arrangement of natural ECM[8]. Scaffold pore size refers to the distance between solid sections of the porous matrix being typically reported as the diameter of circular pores or the major axis for noncircular pores. Pore size seems to be

critical for cell growth and migration and other cellular activities[35,36]. However, the optimal pore size range depends on the materials as well as cell types, making such characteristic difficult to be evaluated. None conclusive result was observed in relation to pore size influence on DTE. However, El-Backly et al[23] concluded that PLGA with pore size between 150-180µm was favorable for vascular formation. Demarco et al[22] verified that DPSC were able to proliferate in PLLA scaffolds, prepared inside of a TS/S, with different pore formats (rounded X cubic) has provided similar results to engineering a dental pulp-like tissue. Considering the lack of studies investigating physical characteristics of scaffolds, it is possible to hypothesize that due to complex nature of biological ECM, the ideal scaffold certainly must combine multiple pore size, fiber diameters and bioactive molecules to generate an organized and functional pulp tissue. The heterogeneity of results obtained with aforementioned scaffolds demonstrated the need for advanced biomimetic scaffolding materials, which had a more predictable behavior.

A new class of material has emerged as an interesting option to be applied in [7,8,21]. The Peptide-Amphiphile (PA) comprises an extremely malleable material's class. PAs self-assemble into three-dimensional networks of nanofibers, and living cells can be encapsulated. They allow the incorporation of the cell adhesion sequence RGD and an enzyme-cleavable site (MMP-2). PAs are also compatible with DSC, allowing cells to spread, proliferate, and differentiate within the hydrogels in vitro[21] and in vivo[7,8]. Due to their rational design, ease to incorporate specific motifs, like RGD and MMP-2 cleavable site, into PA's structure, and injectability make these materials a putative alternative to be used in the translation from the bench to the bedside.

After analyzing the data from selected studies we could observe that the microenvironment cues plays a critical role in pulp-like tissue formation. Particularly, the

contact of stem cells with host dentin is fundamental to achieve a well-organized, functional and even vascularized pulp-like tissue. It is well settled in the literature that the organic matrix of human dentin possess "fossilized" growth factor[37], which are released soon after that tissue is solubilized (e.g. by conditioning with chelating agents, bacterial acids). Promising results with potential for clinical application (Table 4) were obtained using strategies carrying out a previous conditioning over the dentin with Ethylenediamine tetraacetic acid (EDTA). EDTA is a chelating agent able to remove mineralized tissue exposing GFs and collagen fibers, which possess adhesion motifs that improve cell binding via integrin receptors. Moreover, EDTA is able to remove the residual smear layer living open dentinal tubules promoting a cell-friendly environment[7].

TS/S model is a very useful strategy for mechanistic studies. It confirmed the role of GFs present into dentin matrix over the dental stem cells behavior, particularly BMP-2[18,22], and allow to see that PLLA scaffolds are able to create a close contact with adjacent dentin and as a consequence the cells seeded over it have a close contact with dentin and were able to express Dentin Sialoprotein – DSP[19]. Additionally, SHEDs[18] and DPSCs[22] seeded into scaffold without the tooth slice were not able to express the odontoblast markers (DSPP, DMP1 and MEPE). Sakai et al[10] using TS/S, showed that SHEDs can be induced to differentiate into functional odontoblasts and endothelial structures.

The most promising results were demonstrated by two studies using Pas or Poly-D,L-lactide/glycolide[8,9] regenerating pulp-like tissue inside root (dentin cylinder) canal with human dental pulp stem cells. The new-formed tissues were highly vascularized resembling the original dental pulp. The most promising point is that even after the chemical treatment dentin, like in a regular endodontic therapy, cell survival, proliferation and differentiation were not affected.

Conclusions

After this systematic review, taking in account the limitations of our study, we could answer some specific question, as follow:

1. How to make scaffolds more similar to ECM and improve the signaling for cell differentiation? There is not any consensus about the ideal material to be applied in Pulp Tissue engineering. However, after analyzing the available literature, we could notice that a wide range of materials is able to establishing an appropriate and dynamic interface between DSC and the scaffold. The aim strategies used by the researches to improve the crosstalk between DSCs and Scaffolds is the addition of GFs (BMPs-2, 4 and 7, TGF-β1, NCPs (DMP1) to make the chemical composition of materials, synthetic or natural, more similar to ECM. Due to nanofibrillar ECM spatial arrangement, scaffolds able to form nanostructures are a promising material to be applied clinically in DTE. In this sense, the PAs seems to be the putative material to simulate properly the ECM, due to their ease manipulation, ease incorporation of specific motifs like RDG and biocompatibility. In addition, the physical properties of scaffolds like pores distribution, pore size, which are determinant for cell attachment and proliferation.

2. Which variables have capital influence over formation of a well-organized and vascularized pulp-like tissue? We could observe that the environment cues, plays a critical role in pulp-like tissue formation, superimposing even the physicochemical properties of materials used as scaffold. Particularly, to achieve the pulp-like tissue development inside the root canal, the preconditioning of dentin with EDTA seems to be of crucial steps to achieve the properly cell proliferation and differentiation. In addition, the ability of stem cells to express VEGF and differentiate toward endothelial cells are putative aspects to overcome the barrier related to apical foramen being the exclusive access for vascularization

of new-formed tissues inside the root canal. Besides specific points, aiding in translation for clinical application of Tissue Engineering, like the use of Platelet Lysate as substitute of FBS. It would avoid the use of xenogeneic components such undesirable animal growth factors.

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Table 1. Search result at the databases used for searches

Database	Records	Relevant Studies	Excluded
SciVerse Scopus	77	29	48
ISI Web of Science	62	19	43
MEDLINE	41	14	27

Table 2. Full texts evaluated by the two review authors to have its data extracted to evaluation. The studies were ordered by publication date. **Green:** Studies found in SciVerse Scopus; **Orange:** Studies found in ISI Web of Knowledge; **Blue:** Studies found in MEDLINE; **White:** Study not found in specific database

	Study	SciVerse Scopus®	ISI Web of Knowledge®	MEDLINE®
01	Kodonas (2012)	X	X	
02	Chen (2012)	X	X	
03	Zhang (2012)	X	X	X
04	Srisuwan (2012)	X	X	
05	Yang (2012)	X		X
06	Guo (2012)	X	X	X
07	Galler (2012)	X	X	X
08	Galler (2011)	X		
09	Zhang (2011)	X	X	X
10	Demarco (2010)	X	X	X
11	Sakai (2010)	X	X	X
12	Casagrande (2010)	X	X	
13	Huang (2010)	X	X	
14	Gebhardt (2009)	X	X	X
15	Galler (2008)	X		
16	Cordeiro (2008)	X	X	
17	El-Backly (2008)	X	X	X
18	Takeda (2008)	X		
19	Abe (2008)	X	X	
20	Gotlieb (2008)	X		X
21	Prescott (2008)	X	X	X
22	Zhang (2008)	X		X
23	Xu (2007)	X		
24	Deng (2007)	X	X	
25	Huang (2006)		X	
26	Zhang (2005)	X		X
27	Duailibi (2004)		X	
28	Young (2002)	X	X	

Table 3. Full text selected for data extraction (To be published as an on-line appendix of Systematic Review)

Author	Cells/Scaffold	Variables Evaluated/Main Results
Young (2002)	Cells from porcine Third molar tooth buds Polyglycolic/ poly-L-lactic (PGA/PLLA) Collagen Coated (1 x 5 x 5 mm)	**After *In vivo* Implantation**
		Histological Analysis *(H&E, Von Kossa, Goldner staining)* -**20 wks:** Dentin and pulp-like tissue observed and a tissue resembling Hertwig's root sheath epithelia -**25 wks:** Tissue resembling the decalcified enamel. New tooth consisting enamel and dentin-like tissues -**30 wks:** Mineralized enamel, dentin and putative cellular cementum
		Immunohistochemical Analysis *(Amelogenin, Collagen I, BSP immunostaining)* -**20 wks:** BSP immunostaining was observed in the dentin -**25 wks:** collagen type I and BSP in the dentin and odontoblast cells -**30 wks:** Amelogenin present in the ameloblast and in enamel matrix. BSP in the dentin tubules of engineered tissue
		LCM-RT PCR *DSPP and β-actin(Housekeeping Gene)* - Tissue engineered material was positive to DSSP
	Conclusions: Histological, molecular and Immunohistochemical evidence demonstrating the bioengineering of complex tooth crowns resembling those of naturally developing teeth.	
Dualibi (2004)	Cells from rat Third molar tooth buds - PGA and PLGA Scaffolds (1 x 5 x 5 mm)	**After *in Vivo* Implantation – 12 Wks** **Control Groups: (C1)** 7 non-dissociated 4-dpn molar tooth buds implanted as positive controls; **(C2)** 5 unseeded PGA scaffolds; **(C3)** 5 unseeded PLGA scaffolds. **Experimental Groups: (E1)** 8 PGA scaffold implants seeded for 1 hr (ex-vivo); **(E2)** 8 PLGA scaffold implants seeded for 1 hr (ex-vivo)
		- Radiographic Analysis: Experimental groups presented Highly mineralized tissue. C2 and C3 had radiopaque tissue too
		- Histological Analysis: **C1:** Developed well characterized a rat teeth containing all respective tissues **E1** (PGA) and **E2** (PLGA) scaffold cell-seeded formed dentin, enamel and pulp tissue **Goldner's Stain** (dentin and bone blue, newly formed enamel matrix red, and mature enamel matrix gray)**: C1** exhibited blue-stained dentin, red-stained enamel, and gray-stained mature enamel. **E1** generally exhibited more mature enamel that stained gray, while **E2** generated both immature and mature enamel that stained reddish to gray.
		- Immunohistochemical Analysis: Amelogenin in bioengineered enamel. **C1** exhibited positive Amelogenin expression in ameloblasts and in demineralized enamel. Bioengineered enamel grown on both **E1** and **E2** scaffolds exhibited positive staining for amelogenin
	Conclusion: PGA and PLGA scaffold have not shown difference in its ability to support the growth of dental tissues.	

<table>
<tr><td rowspan="3">Zhang (2005)</td><td rowspan="3">Primary Culture of Rat Dental Pulp Cells (RtDPCs)
-
Hydroxyapatite–tricalcium phosphate (HA–TCP – 60:40) and Composite titanium fiber mesh(Ti) scaffolds</td><td colspan="2">In Vitro Analysis</td></tr>
<tr><td colspan="2">Immunohistochemistry (STRO-1): Approximately 5% of cells showed positive staining</td></tr>
<tr><td>Culture onto smooth surfaces (2x10^4)
Days 1, 3, 5, 10, 14, 21, 28</td><td>Culture onto 3D Scaffold – (HA–TCP) and Ti – (1x10^6)
After 0 (control), 1, 2, 4, 6, 8 wks analysis were made</td></tr>
<tr><td>von Kossa: after 14 days were detected nodules positive in cells cultured into induction media
Proliferation rate: RtDPCs reached confluence after 5 days
ALP activity: reached a peak at day 14
SEM: cell attached after 1 day of culture
RT-PCR: DSPP expression (day 5 until 28); OC (Day 3); Col I (All specimens)</td><td>Proliferation rate: 0 = DNA values of the HA–TCP>Ti (suggests that more cells had attached to HA–TCP). 1 = Ti>HA-TCP. Almost no DNA was detected on wk 8
ALP activity: Increasing until wk. 6 and declined slowly until wk. 8
SEM: Cells connected with each other, and produced collagen on both materials, after 1. Mineralized nodules present after 2wks. on HA-TCP and on both scaffolds after4 wks.
RT-PCR: DSPP mRNA inexistent on control was expressed after wk 2 in both material and its expression increased until wk 8</td></tr>
<tr><td colspan="3">Conclusion: The use of titanium or calcium phosphate ceramic exerted little influence on the differentiation pattern of the cells toward an odontoblastic phenotype, as induced by dexamethasone. Rat dental pulp stem cells offer a valid model for studying the regeneration of dental tissues. Author’s Words</td></tr>
<tr><td rowspan="3">Huang (2006)</td><td rowspan="3">DPSC (by enzyme or outgrowth methods)

Collagen Gel and dentin (used as substrate)</td><td colspan="2">In Vitro Analysis</td></tr>
<tr><td>- Cell proliferation onto both substrates:</td><td>Over Dentin: Cells proliferate minimally
Into Collagen Matrices: Higher cell proliferation</td></tr>
<tr><td colspan="2">- Gene Expression (DSPP, Col I and Col III): Cells stained
- Western Blot (Anti-Dsp): Cells stained
- SEM: Cell onto dentin exhibited odontoblast morphology
- Collagen Gel-contraction assay: Cells were potent on scaffold contraction (34%)</td></tr>
<tr><td colspan="3">Conclusions: Direct contact of pulp cells with mechanically and chemically treated dentin may induce pulp cells differentiation towards odontoblasts with processes extending into dentinal tubules, Collagen matrix alone may not be a suitable scaffold for pulp tissue engineering.</td></tr>
<tr><td rowspan="3">Xu (2007)</td><td rowspan="3">Human DPSCs Periodontal cells (PC)
-
PLLA/nano hydroxyapatite (HA)</td><td colspan="2">In Vitro Analysis</td></tr>
<tr><td colspan="2">- SEM (PLLA/HÁ): Thefiberdiameterrangedfrom270to330nm</td></tr>
<tr><td>DPSC and PC behavior when seeded onto PLLA/HA</td><td>- Morphology: changed the round shape to elongate and spindle-shaped
- Outgrowth: The cells exhibited classical contact guidance by growing parallel to the PLLA fibers.</td></tr>
<tr><td colspan="3">Conclusion: The suitability of PLLA/HA scaffolds for the DPSCs, PCs, culture was confirmed. The Nanofibers highly supported the DPSC and PC and improved dental ingrowth</td></tr>
</table>

Deng (2007)	Human Dental Pulp Stem Cells (DPSC) PLLA/ Multi-walled carbon nanotubes (MWNTs)/ hydroxyapatite (HA) nanofibrous scaffolds	***In Vitro* Analysis**		
		- SEM (PLLA/MWNT/HÁ): the average diameter of hybrid nanofiber was similar to that of PLLA/HÁ (Xu et al., 2007); DPSCs were well attached on all the scaffolds and the cells changed their original round shape to elongated and spindle-like shape.		
	Conclusion: Like PLLA/HA nanofibrous scaffolds (Xu et al., 2007) The PLLAMWNT/HA nanofibers highly supported the DPSC and PC and improved dental ingrowth			
Zhang (2008)	Primary Culture of: Rat BMSCs Rat DPSCs Human BMSCs Human DPSCs HA/TCP Ceramic seeded with the four cell used to compare their behavior	***In Vitro* - Weeks 0, 1, 3 and 5**		
		Cell Attachment (Control Medium)	**Rat:** 42% of the BMSCs attached onto HA/TCP; 9% of DPSCs attached **(p<0.01).** **Human:** 26% of the BMSCs viable; 46% of DPSCs attached. **No statistical difference**	
		Osteogenic Medium – **Cell Proliferation**	**Rat:** BMSCs > DPSCs – Wks 0, 1, 3 (P<0,05)	**Human:** BMSCs > DPSCs (P<0,05)
		Osteogenic Medium – **ALP Activity**	**Rat:** BMSCs < DPSCs – Wks 0, 1, 3 (P<0,05)	**Human:** BMSCs < DPSCs Wk 0, 5 (P<0,05)
		Osteogenic Medium – **SEM**	Abundant cell growth and mineralization of ECM for all cells. A smaller amount of cells was detected on the scaffolds loaded with human DPSCs	
		Osteogenic Medium – **qRT-PCR**	**OC:** BMSCs > DPSCs **Runx2:** was higher for hBMSCs (peak) followed for rBMSCs in week 3 **DSPP:** was not found in any BMSCs. For both types of DPSCs, the DSPP peak value was found at week 3 with significant decrease at wk 5	
		***In Vivo* - After 5 and 10 wks**		
		Histological Analysis (*Methylene blue/basic fuchsin*_ All the retrieved implants with or without loaded cells were surrounded by a thin fibrous capsule. None of the ceramic implants showed visual signs of degradation. - **rBMSC:** bone-like tissue formation, the majority at the periphery of scaffold. After 5 wks was possible to observe mature bone with significant increase in bone-like tissue formation after 10 wks. - **rDPSC:** In wk 5 no specific bone formation was observed. Neither in wk 10 - **hBMSC:** no bone formation was observed. At wk. 5 a fibrillar ECM was observed and in wk 10 a thicker collagen-like matrix was observed on the surface of pores**;** **- hDPSC:** no evident bone formation was detected at week 5. A fibril-like ECM was found in the samples of week 10.		
		qRT-PCR	**OC – Runx2:** BMSCs > DPSCs; **DSPP:** was not expressed in BMCSs	
	Conclusion: Sintered HA/TCP supported the attachment, growth, and differentiation of all four types of cells in vitro and in vivo.. Only DPSC were able express DSPP, in vitro an in vivo, showing its potential to differentiate toward odontoblast lineage. This study elucidates clearly effect of combination cell+scaffold			

Prescott (2008)	DPSC (2) - Collagen - DMP1 was used as Morphogen	***In Vivo* –** Tooth Slice (38) adapted. The pulp chamber ground was maintained to simulate the perforation at the furcation local. 5 groups (n=4) – G1=Grey MTA; G2=Scaffold collagen alone; G3=Collagen+DMP1; G4=Collagen+DMP1+DPSC; G5=Collagen+DPSC **Histological Analysis -** *Hematoxylin-Eosin, Safranin-O, Von Kossa, And Masson's Trichrome* **G1:** No cells, collagen matrix, blood vessels, or calcified tissues; **G2:** Only degrading Collagen Scaffold was stained with Masson Trichrome; neither pulp-like replacement tissue was saw **G3:** Great number of nucleated cells and little ECM into perforation; lack of cellular organization **G4:** Signs of tissue regeneration with degradation of collagen matrix and presence fusiform and endothelial cells forming a new ECM. Was possible to detected blood vessel and collagen into the perforation site. Clear tissue organization **G5:** Similar to G2
	- DMP1 induced the DPSC differentiation towards matrix-synthesizing cells - Was confirmed the potential use of Stem Cell-Based Therapies to evaluate tissue regeneration mechanisms and hard tissue formation into DPC	
Gotileb (2008)	SHED (1) - D,D-L,L-polylactic acid (OPLA) and collagen scaffolds; BMP-2 and TGF-β1 were used as Morphogen	***In Vitro* evaluation by Ultrastructural Scanning Electronic Microscopy (SEM)–**105 human Pre-Molar (hPM) with single root canal (length 16mm). The constructs were divided in seven experimental groups **- G1:** Without any Scaffold; **G2:** SHEDx10^6; **G3:** OPLA+SHEDx10^6; **G4:** Collagen+SHEDx10^6; **G5:** Collagen+SHEDx10^6+BMP-2; **G6:** OPLA+SHEDx10^6+BTGF-β1; **G7:** OPLA+SHEDx10^6+β-Glycerophosphate. **hPM cultured (24 well plate) and evaluated after 1, 7 and 14 days (n=5)** **- Cell Attachment:** SHED (G2) attached to root dentin; All experimental groups showed SHED attachment onto respectively Scaffolds **- Attachment of a dental pulp construct to root dentin:** No obvious differences in the cell activity between the experimental groups. All of the dental pulp constructs had some contact with the root canals (frequently < 50%). OPLA scaffolds appeared to attach more completely with the root canal dentin than did the collagen scaffolds. The constructs adherence was better in the coronal portion of hPM root canal.
	Conclusion: The results found by the authors support that is possible to use dental pulp constructs as biological endodontic therapy. However the detection of SHED on parts of the root canal wall is not a conclusive presumption for the potential of this technique to develop reliable vascularization and to regenerate the root pulp tissue.	
Abe (2008)	Primary Culture of Apical Pulp Derived Cells (APDCs) - Porous Hydroxyapatite (HA) Mineralization media: rh-BMP2	***In Vitro* – ADPCs at 3rd passage (2.5x10^5) – ADPC+HA cultured for 10 days into mineralization media** **Mineralized cell differentiation (ALP – up to 3 wks):** cells showed time-dependent ALP activity **SEM analysis (**1, 2, 3 wks after seeding**):** APDCs had disseminated well on the scaffolds ***In Vivo* implantation ADPC+HA (5x5x2mm) into dorsum of null Rats - ADPCs at 3rd passage (5x10^5)** **Histological Analysis(HE, Masson Trichrome and Immunohistochemical staining):** The APDCs generated ectopic bone-like tissue and dentin-like tissues (dentin-like matrix, pulp-like tissue), and odontoblastic cells. Bone-like tissue was more abundant than dentin-like tissue
	Conclusion: APDCs formed both bone and dentin in vivo. The study suggests that the human tooth with an immature apex is an effective source of cells for bone and dentin regeneration	

Takeda (2008)	**DPSC from 15-25 yrs. old volunteers:** - Crow-completed (hDPSC-C) - Root-Formative (hDPSC-R) - Root-Completed (hDPSC-R) - hMSCs (20 yr. old individual) - Calcium Phosphate Scaffold	**In Vitro – Cells cultured in mineralization, odontogenic and adipogenesis induction media** **Isolation and characterization:** 48 ± 30 colonies/104 (hDPSC-C) and 40 ± 35 colonies/104 cells (hDPSC-R); both hDPSC were able to differentiate in adipocyte, neural and odontoblastic-like cell. hDPSC-C were more proliferative than hDPSC-R **ALP Analysis and von Kossa staining:** both cells were ALP+ and von Kossa showed a calcified matrix. After 10 passages (P10) ALP activity decreased significantly **Population Doublings:** 42 ± 2.8 hrs (hDPSC-C) and 65 ± 6.5 hrs (hDPSC-R) - P = 0.0001. **DNA array:** genes up or down-regulated more than 5 fold when P4 and P10 were compared were selected — **P4:** 642 genes down-regulated 2 fold; **P10:** 719 genes down-regulated 2 fold **qRT-PCR:** WNT16 mRNA expression increased significantly with an increase in passage number in all the samples tested. TPD52, ITPR1, and TLR4 mRNA decreased with passage number. TLR4 decrease was slower in hDPSC-R. **In Vivo analysis – Cells seeded onto scaffolds by 7 days before implantation in mice. Implants removed after 8 and 15wks.** **Frozen non-decalcified sections (H&E):** **8wks:** both hDPSC started to generate a matrix on the scaffold surface. Cells had lost that capacity after P10 **15wks:** both hDPSC had forms dentin and pulp-like tissues
	Conclusion: Were isolated hDPSCs from developing third molars, extracted before eruption. hDPSC-C were more proliferative than hDPSC-R. The hDPSC-Cs were capable to generate dentin and pulp-like tissue when implanted *in vivo* onto calcium phosphate scaffolds. However, these characteristics were lost in long-term culture, with a change in their gene expression profile.	
El-Backly (2008)	Rabbit Dental Pulp Stem Cells (RDPScs) - 50/50 poly(lactic-co-glycolic acid) using two different particle size (150-180µm and 180-300µm)	***In Vitro* analysis – G1: 150-180µm and G2: 180-300µm** **- SEM:** G1: pore size ranged from 8 to 250µm; G2: pore size ranged from 30 to more than 300µm. Higher interconnectivity was observed in two groups. - **Mercury Intrusion:** G1: Total pore area was 71.208 $m^2 g^{-1}$. Median pore diameter (volume) was 0.0986 mm. Porosity: 37.02%; G2: total pore area was 43.466 $m^2 g^{-1}$. Median pore diameter (volume) was 0.0324 mm. Porosity: 20.56%. - **Colony-Forming:** 47–53 colonies per 10^4 cells **- Immunophenotyping:** immunoreactivity when treated with the marker p75 nerve growth factor receptor 5 (CD271). **- Culturing RDPScs onto scaffolds (1 – 12 days):** Porosity has increased as the material degrades. **- Phase contrast micrographs:** G1: cell attachment and proliferation into and around the pores, degradation was apparent at the scaffold margin; G2: similar to G1 **- SEM:** Was demonstrated efficient cell colonization and attachment onto the both scaffolds evaluated. Surface degradation evident by the new pores detection
	***In Vivo* –**Histological results of the subcutaneously transplanted seeded and unseeded scaffolds	
	Was possible to observe an increase in the number of blood vessels and capillaries in all specimens, however the experimental constructs, which were seeded with stem cells showed a more pronounced vascularization than the control groups (Scaffolds only)	

	G1 Control - Wk 2: dense fibrous scar tissue formed by collagen fibers; **Wk 6:** no sign of fibrous tissue capsule. Increase in vascularization	**G2 Control** (Osteodentine-like tissue more evident) - **Wk 2:** dense fibrous tissue capsule demarcating the transplant area; close to the capsule wall budding capillaries; **Wk 6:** very thin small capsule seeming to contain fibrous tissue for replacement of the degraded scaffold
	G1 Experimental - Wk 2: presence of a well-circumscribed structure related to the capsule wall lined by well-organized cells in several layers. Within the lumen of this structure, there is a secretory-like product; **Wk 6:** A variety of dentin-like structures could be identified and all of them close to blood vessel. Several well circumscribed areas filled with pink material. Around these structures, there was increased cellularity; presence of cells in tubular-like arranged patterns	**G2 Experimental - Wk 2:** a thin and dense fibrous capsule. Numerous blood vessels surrounded by a dense cellular pink fibrous tissue similar to dentine/pulp complex. Characteristic vertical arrangement of columnar cells especially adjacent to the walls of blood vessels. Structure similar to early stages of dentin-pulp complex; **Wk 6:** very thin lining remaining from the capsule saw at 2 weeks. Columnar cells arranged in tubular-like structures. There are also well-circumscribed elongated structures consisting of organized vertically arranged parallel tubules similar to a dentine-like appearance (Author's words)
	Conclusion: Due to complex nature of biological ECM the ideal scaffold certainly would combine multiple pore size, fiber diameters and inducers molecules to generate an organized and functional pulp tissue.	
Cordeiro (2008)	**SHEDs** (Miura et al., 2003) **SHEDs-LacZ** **HDMECs** (human dermal microvascular endothelial cells) - poly- L-lactic acid (PLLA) using salt (250–450 µm) as porogen	**In Vivo –** Tooth Slice/Scaffolds (T-S/S): slice of human teeth with the space corresponding to pulp tissue filled with a scaffold, which had stem cell seeded onto its surface **(n=6) - T-S/S1: 8x10^5 SHED; T-S/S2: 0.8x10^5 SHED + 7.2x10^5 HDMEC; T-S/S3: Empty PLLA Scaffold; Positive Control: Slice of human tooth without pulp tissue removing. SHED-LacZ and SHED-LXSN** were used to evaluate the fate of transplanted cells. Cells were suspended in 1:1 matrigel:culture media solution. After 14 and 28 days implants were retrieved to carry out the analyses.
		- Histological Analysis: PLLA Scaffolds occupied the pulp chamber space. In the both time point pulp-like tissue was generated inside the T-S/S.
		- Cells Lining T-S/S Dentin: the co-implantation (SHED+HDMEC) did not have a significant effect on the cells' density (P>0.05)
		- Immunohistochemistry — **Anti-Human DSP**: cells adjacent to T-S/S pre-dentin(PD) similarly to positive control **Anti-Human Factor VIII**: SHED+HDMEC cells did not result in a significant increase in microvessel density, when compared with the implantation of SHED alone.
		- **TEM:** The cells showed odontoblast's morphologic characteristics; the eccentric polarized position of the nucleus at the basal part of the cell body; several gap junctions at the cell contact area; well-developed rough endoplasmic reticula, a well-developed Golgi's complex, (typical of odontoblasts).
		- SHED-LacZ: LacZ positive cells were observed adjacent to PD; innumerous blood vessel were lined with positive LacZ Cells
	Conclusion: This work suggests that exfoliated deciduous teeth constitute a viable source of stem cells for dental pulp tissue engineering. Besides had showed remarkable evidences elucidating the ability of SHED to generate pulp-like tissue and endothelial structures when implanted *in vivo.* Therefore, these cells form a functional vasculature as well as connective tissue secreting cells of the soft and hard tissues of the tooth. Besides, these results suggest that the co-implantation of endothelial cells might not be a necessary step for the engineering of tissues with SHED.	

<table>
<tr><td rowspan="3">Galler (2008)</td><td rowspan="2">DPSCs and SHEDs (1, 2) - Peptide-amphiphile (PA) hydrogel scaffolds with specific RGD sites MMP-2 susceptible zone</td><td colspan="2">In Vitro analysis of cell (2x 10^6) differentiation when seeded onto Pas, which were separated in three groups – G0 (Control): PA without any induction media; G1 (βGP+dex): mineralization induction media (MIM) charged with β-glycerophosphate and dexamethasone; G2 (KPh+dex): MIM charged with KH_2PO_4 and dexamethasone
- Cell Proliferation: SHED>DPSC into PA; βGP+dex allowed a slightly in the cell proliferation of both cell lines; KPh+dex had attenuated the cell growth.
- Measurement of DNA content (3, 7, 14 and 28 days): After 28 days, SHED number was close to 4x10^5, while DPSC did not exceed 1.5x10^5 cells per gel.
- ALP Activity: In SHEDs is increased with βGP+dex and reduced with KPh+dex; DPSC happen the opposite</td></tr>
<tr><td>- qRT-PCR (Collagen I and III, ALP, BSP, OC, Runx2, and DSPP):</td><td>- DPSCs and SHEDs increased those genes expression until day 28
- DPSC into KPh+dex did not express DSPP
- SHED into βGP+dex improved the expression of genes involved in - matrix deposition (Col I and III) and osteoblast differentiation; discrete increase in DSPP</td></tr>
<tr><td colspan="3">Conclusion: PA nanofibers are compatible with SHED and DPSC. Cells spread, proliferate, and differentiate within the hydrogels. SHED appear more adept for soft tissue regeneration, while DPSC have a greater potential for terminal differentiation and subsequent mineralization.</td></tr>
<tr><td rowspan="3">Gebhardt (2009)</td><td rowspan="2">DPSCs (2), PLSCs and L929 (Control Cell Line)
-
Three Scaffolds:
- Open-cell D, D-L, L-polylactic acid
- Bovine collagen
- Calcium phosphate bioceramic</td><td colspan="2">In Vitro Analysis of DPSCs, PLSCs and L929s (x10^6) survival in constructs after 3, 7 and 14 days</td></tr>
<tr><td>Histological Analysis</td><td>Cell Viability (Neutral Red Dye <0.05): type of scaffold was determinant for cell survival (p=0.0001). Polymeric and collagen scaffold showed a great number of DPCSs and PLSCs. No significant difference in cell survival onto this two scaffolds after 14 days (p=0.1453). L929s were similar to DPSCs and PLSCs

Cells Location: Polymer and collagen scaffolds showed predominantly evenly distributed forming-colony cells. Calcium phosphate scaffolds had a high occurrence of single or no cells,</td></tr>
<tr><td colspan="3">Conclusion: Polymer and Collagen constructs showed a great potential to be used in Dental Tissue Engineering</td></tr>
</table>

<table>
<tr><td rowspan="6">Huang (2010)</td><td rowspan="6">SCAPs (39)

DPSCs (2)

Poly-D,L-lactide/glycolide Inserted in tooth fragments simulating root canal</td><td colspan="2">In Vitro – SCAPs or DPSCs (5x10^5) were isolated and characterized (CD14-PE, CD34-PE, CD45-FITC, CD73-PE, CD90-FITC, and CD105-PE). Submitted to multilineage differentiation (Odonto/Osteogenic, Adipogenic, Neurogenic and Myogenic). Both cell seeded onto scaffolds has their morphology and attachment properties evaluated after 10 days or up to 8 wks.</td></tr>
<tr><td colspan="2">SEM: Both cells attached well to PLG Scaffolds. After 8 wks cell laid down a fiber matrix</td></tr>
<tr><td>Histological Analysis</td><td>Blank PLG: Colorless
DSPC or SCAP: eosin staining seen in the DPSC-seeded PLG represented the cytoplasm of cells and extracellular matrix</td></tr>
<tr><td colspan="2">In Vivo: Section of radicular human teeth – RHT - (~6-7mm) were enlarged and sealed with MTA in one end. RHT were treated with EDTA (19%) Citric Acid (17%) Betadine and NaOCl (5.25%). After the cells previous seeded onto scaffold were inserted into RHT to form the “Tooth Construct” (RHT+Scaffols+DPSC/SCAP). The control root fragment did not receive any cell-seeded PLG in the emptied root canal space.</td></tr>
<tr><td>Histological Analysis</td><td>Tooth constructs seeded with both cell were filled with pulp-like tissue after 3-4 months. The entire pulp-like tissue was vascularized with uniform cellularity showing well-aligned odontoblast-like cells with polarized cell bodies lined against the new formed mineralized dentin-like tissue</td></tr>
<tr><td>Immunohistochemistry</td><td>Suggest that the differentiated odontoblast-like cells were responsible for the production of the calcified tissue. Cells were ALP, DSP, BSP and CD105 positive</td></tr>
<tr><td>Human Mitochondria</td><td>Have confirmed the human origin of cell into regenerate pulp-like tissue</td></tr>
<tr><td colspan="3">Conclusion: It was the first in vivo evidence of de novo synthesis of vascularized human pulp/dentin-like tissue into a root canal segment using SCAPs and DPSCs, which have differentiate itself in odontoblast-like cell able to secrete dentin matrix in close and physiologic relation with dentin walls.</td></tr>
<tr><td rowspan="6">Casagrande (2010)</td><td rowspan="6">SHED (1)
-
PLLA into Tooth Slice/Scaffold (TS/S)</td><td colspan="2">In Vitro Analysis to verify the role of BMP-2 fossilized into dentin matrix in SHED differentiation towards odontoblast Phenotype. Tooth Slice/Scaffolds (TS/S) submitted to different conditions prior cell seeding: TS/S1- 5.25% NaOCl for 5 days at 4°C; TS/S2- 10% EDTA (pH 7.2) for 1 min at 4°C; TS/S3- untreated control TS/S; S4- control PLLA scaffolds without the tooth slice(TS) – SHED (5x104): 0, 7, 14, 21 and 28 days.</td></tr>
<tr><td>Western Blot</td><td>SHED expressed BMPR-IA, BMPR-IB, and BMPR-II (BMP receptors)</td></tr>
<tr><td rowspan="3">RT-PCR
(DSPP, DMP1, MEPE)</td><td>rhBMP-2: strong expression of Odontoblast markers (OM)</td></tr>
<tr><td>rhBMP-7: weak expression of DMP1</td></tr>
<tr><td>Untreated: Negative for all OM</td></tr>
<tr><td>RT-PCR
(DSPP, DMP1, MEPE)
Into TS/S up to 28 days</td><td>OM expression observed in TS/S2 and TS/S3. In TS/S1 (where proteins were denatured by NaOCl) and S4, OM were not expressed
Additionally, the authors applied neutralizing antibodies to BMP-2 and BMP-7. Blockade of BMP-2 receptor avoid dentin to induce OM by SHED</td></tr>
</table>

		WST-1	SHED seeded onto S4 showed a significantly higher proliferation rate) followed TS/S1, TS/3 and TS/S4 after28 days of culture
		***In Vivo* implantation of TS/S1, 2, 3 and S4**	
		- RT-PCR: The results were quite similar to those obtained *in vitro*. Notably the pre-treatment of TS/S dentin with EDTA for 1 min was responsible for the high expression of OM by SHED 28 days after *in vivo* implantation**.**	
		- Histological Analysis: The tissues generated within TS/S2 resembled more closely the morphology of normal dental pulps, as compared with TS/S3.	
	Conclusion: dentin-derived morphogenic signals, and particularly BMP-2, are necessary and sufficient to induce the differentiation of stem- cells towards odontoblasts.		
Sakai (2010)	SHED (1) - PLLA into Tooth Slice/Scaffold (TS/S)	***In Vitro* analysis – TS/S previously treated with 10% EDTA (pH 7.2) for 1 min at 4°C were randomly assigned to: TS/S1 –** TS/S+SHED ($5x10^6$); **TS/S2 –** TS/S without SHED**; TS/S3 –** TS/S without SHED and without Scaffolds; **S4 –** Control Scaffold+SHED without TS; **TS/S5 –** Control TS/S (Gonçalves et al., 2007) **– 0, 7, 14, 21 and 28 days supplemented with 0 or 50 ng/mL rhVEGF$_{165}$.**	
		Gene Expression VEGFR1, VEGFR2, PECAM1, and VE-Cadherin (1-28 days) of VEGF treatment	After 28 days in culture, SHED cultured in the tooth slice/scaffolds in the presence of VEGF expressed all markers of endothelial differentiation evaluated
		- Capillary sprouting in 3-D collagen matrices (40)	SHED cultured with VEGF showed higher numbers of capillary sprouts than controls
		- Hard Tissue Formation in a Porous	Silencing of VEGFR-1 expression in SHED was determinant in down-regulation of capillary sprouting
		***In Vivo* Analysis –** Specimens, at the same conditions applied *in vitro, w*ere transplanted into the mices' dorsum. SHED-LacZ and SHED-LXSN were used to evaluate the fate of transplanted cells.	
		- Tetracycline Injection (Dentin deposition by SHED)	Confocal microscopy revealed 4 clearly defined fluorescent lines in TS/S1 corresponding to intraperitoneal injections.
		- RT-PCR (DSPP, DMP1) - OM After 32 days	Tissues developed within tooth slice/scaffolds seeded with SHED expressed OM. Additional verification of SHED differentiation was performed by DMP-1 immunohistochemistry, DMP-1 Western blot, alkaline phosphatase staining in vitro
		- Histological Analysis	The new tissue that was generated in the pulp chamber of TS/S seeded with SHED showed dentinal tubules and a clearly defined pre-dentin layer
	Conclusion: This study provided the first concrete evidence that SHED are able to differentiate towards odontoblastic cells capable to form pulp-like tissue and secreting dentin matrix. The tetracycline staining demonstrated unequivocally that odontoblast-like cells originated from SHED, seeded onto TS/S, have deposited new dentin. The beta-galactosidase-positive blood vessels were found close to non-stained (host) blood vessels. These findings demonstrate that SHEDs are able to differentiate into functional blood vessels able to connect with the host vasculature.		

Demarco (2010)	DPSC (2) - PLLA into Tooth Slice/Scaffold (TS/S) Salt x Gelatin spheres (250–450 µm) as porogen	***In Vitro* Analysis – TS/Ss were sieved with Sodium Chloride (250-425 mm) or spheres of gelatin. TS/S previously treated with 10% EDTA (pH 7.2) for 1 min at 4°C. PLLA Scaffolds without TS (control). DPSC ($5x10^4$) were seeded onto TS/S**	
		WST-1	DPSCs proliferation was higher when cells were seeded into scaffold-only
		RT-PCR (DSPP, DMP1, MEPE) 0, 7, 14, 21, 28 days	OMs were expressed by DPSCs cultured within tooth slices but not in the scaffold-only. Human odontoblast expressed the three OM
		***In Vivo* transplantation of TS/S seeded with DPSCs ($6x10^5$) into immunodeficient mice**	
		- Histological Analysis: after 28 days a pulp-like tissue was observed in TS/S and control Scaffolds. TS/S pulp-like tissue showed a better organization resembling dental pulp **- Immunohistochemical Analysis:** Factor VIII positive tissues - **Gene Expression:** Odontoblast Markers were expressed by tissues formed into TS/S after 28 days implanted in mice	
		Both porogens used showed similar results regarding cell proliferation, differentiation and pulp-like tissue formation	
	Conclusion: Morphogenic cues originated from the three-dimensional microenvironment influence the differentiation of dental pulp stem cells into odontoblasts. Particularly, dentin related factors appear to play a major role in odontoblastic differentiation.		
Zhang (2011)	Isolated Human Dental Pulp Stem Cells (hDPSCs) - Bone Marrow Cells (BMCs) Silk (RGD or DMP1) Scaffolds 500 x 1000µm Pore Size Constructed by hexa-fluoro-2-propanol **(HFIP)** or Aqueous **(AQ)** method	In Vitro evaluation of cells ($4.5x10^5$) on Silk Scaffolds – Negative Control: Scaffold alone and Cell-Seeded Scaffold both in induction medium. **8 experimental Scaffolds** (Method-Pore Size-Growth Factor)**: HFIP-500-RGD; HFIP-1000-RGD; HFIP-500-DMP1; HFIP-1000-DMP1; AQ-500-RGD; AQ-1000-RGD; AQ-500-DMP1; AQ-1000-DMP1;**	
		Fluorescence Calcein Content: Detected only in Cell-Seeded Silk Scaffolds	
		In Vivo implantation – DPSCs/BMCs + Silk Scaffolds – 6, 18 and 25 Wks	
		- Histological Analysis	**6 Wks:** Silk (AQ) degradation and blood vessel formation; no calcified tissues – HFIP without degradation **18 Wks:** Only HFIP observed; AQ degraded **25 Wks:** Both HFIP and AQ degraded ($p<0.05$)
		Collagen was predominant HFIP; Blood vessel formation p/.0.05; Cell seeding did not changed the degradation rate; no hard tissue formation in DMP1-charged silk scaffolds; no significant difference soft tissue formation was detected between RGD or DMP1-charged silk scaffolds	
	Conclusion: AQ degraded much faster than the HFIP; Diameter pores demonstrated that although the scaffold pores appeared to support the formation of soft tissue; HFIP based Silk scaffolds seeded with human DSPs may be useful for soft tissue, dental pulp regeneration		

Galler (2011)	**DPSCs** (2) - **(PA)hydrogel** VEGF, TGF-b1, and FGF2	***In Vitro* Analysis – Dentin Cylinders – DC – (4mm height; inner diameter of 1.5 mm – Group A: 10 minutes in 5.25% NaOCl; Group B: 10 min in 5.25% NaOCl + 2 min 1% EDTA; Negative control: without dentin conditioning**	
		SEM:	NaOCl = Surface of Root Canal covered with debris, Smear Layer was not removed
			EDTA = Surface without Smear Layer, Dentin tubules exposed
		In Vivo Analysis – Negative Control; Group A and Group B – 6 Wks	
		For groups with GFs and Cells was possible to observe a soft and vascularized tissue; PA Degraded and substituted by dental pulp like cells	
		Histological Analysis:	**Control:** no cell migration **Group A:** multinucleated cells creating resorption lacunae; odontoclastic activity **Group B:** intimate association between implanted cells and dentin matrix; Flat cells projecting cellular process inside de dentin tubules
		Immunohistochemistry (Dsp):	**Group A:** Negative **Group B:** Positive
	Conclusion: Dentin conditioning considerably influences DPSC fate when seeded in close proximity to dentin. This information might be critical for optimized strategic planning for future regenerative endodontic treatment.		
Galler (2012)	**DPSC** (2) - **(PA)hydrogel** cell adhesive (RGD), enzyme-cleavable (MMP2) - **VEGF, TGF-β1**, and **FGF2**	***In Vitro***	
		DPSCs in 2D Cultures	**GFs release kinetics (1, 2, 3, 5, 7, 10, 14 days):** GFs were released after 10 days and had their kinetics retarded by heparin **GFs x Cell morphology and proliferation:** In regular medium the cells exhibited their spindle-shaped morphology. FGF2: cells more elongated > TGF-β1 ($P<0.05$)
		DPSCs into PA-MDP	**Cell Morphology after 14 days (Confocal):** Cells elongated and stretched out in contact with neighboring cells producing collagen as their own ECM to replace the scaffold**.**
		***In Vivo* Transplantation: MDPs into Dentin cylinders – DC (**height: 3mm; inner diameter: 1mm; outer diameter: 3mm) – Canal enlarged by a diamond burr. Before cell seeding – 5% NaOCl (10 min.) 17% EDTA (2min.) Groups designed for the authors: **(A)** MDP, no GF, no cells (baseline control); **(B)** MDP, GF, no cells (cell-free control); **(C)** MDP, no GF, DPSC (GF-free control); **(D)** MDP, GF, DPSC (experimental groups charged with VEGF, TGF-β1, and FGF2). Analysis cares out after 5 weeks of implantation	
		Histological Analysis (H&E and Masson's Trichrome):	**A:** MDP matrix and DC into fibrous capsule **B:** GFs attracted cells **C:** Cells do not proliferate enough to fill the root canal segment; complete degradation of MDP **D:** formed a vascularized tissue resembling the original dental pulp
		Immunohistochemical analysis (DSP and Factor VII): cell seeded into MDP with GFs (D) expressed DSP and Factor VIII	
	Conclusion: Subcutaneous transplantation of the hydrogel within dentin cylinders led to the formation of a vascularized soft connective tissue similar to dental pulp. These data support the use of this novel biomaterial as a highly promising candidate for future translational research		

Srisuwan (2012)	DPC - Collagen I Scaffold BMP-4 and FGF2	***In Vivo* (2, 4, 6 and 8 Wks) – Rats divided in 5 groups - G1:** DPC, collagen only matrix, placed in a silicone chamber; **G2:** collagen matrix + FGF2 and BMP-4 , in a silicone chamber; **G3:** DPC, collagen matrix, placed in a dentin insert in a silicone chamber; **G4:** DPC, collagen matrix + FGF2 and BMP-4, in a dentin insert; **G5:** DPC, collagen matrix + FGF2 and BMP-4 in a chamber containing dentin fragments	
		Histological Analysis:	**- G1 and G2:** The constructs consisted of vascularized connective tissue growing around the pedicle. No difference between the groups with or without BMP-4 and FGF2; **- G3 and G4:** Less vascularized connective tissue was observed around the pedicles in the constructs with rigid dentin inserts. Increase inflammatory cells; **- G5:** Increase in construct size and tissue formation. Tissue surrounding the dentin fragments healthy and vascularized
		Immunohistochemical Analysis:	**- G1 and G2: s**tained strongly for DSPP **- G5:** positive for DSPP
	Conclusions: This study demonstrates the potential of an arteriovenous loop and vascular pedicle tissue engineering model to provide blood supply for dental pulp cells and thus evaluate their behavior in dental pulp-like tissue regeneration. It can be a putative strategy for future development of an alternative treatment using these techniques.		
Guo (2012)	Rat Dental Follicle Cells (DFCs) and Rat BMC - Treated Dentin Matrix (TDM) Scaffold	***In Vitro* – Every stem cell-characteristics were minutely evaluated by the authors**	
		Immunocytochemistry – DFCs were positive for Notch-1, CD146, ALP, COL-1, COL-3 and OCN which are all markers of DFCs; Positive for CD-146; Cells were able to differentiate in Osteogenic, adipogenic and neuronal lineage. .	
		***In Vivo* Roots generation –** TDM as an inductive microenvironment for the pulp-dentin complex. Fresh alveolar fossa as inductive microenvironment for cementum-periodontal ligament complex. TDMs function as scaffolds - 4 Wks -**Experimental Group (EG):** TDM + Polypropylene Tubules (PT) + DFC**; Cellular Control Group (CCG):** TDM + PT + BMC; Microenvironment Mineralization Control Group (MCG) **–** Cranial fossa of skulls: Microenvironment Non-Mineralization Control Group (NMCG) – Omental pockets.	
		- Histological Analysis	EG: generation of a pulp-dentin complex, a cementum-like layer, and a PDL inserted into the alveolar bone CCG: no tooth structures detected NMCG: complete dentin tissue regeneration MCG: mature mineralized matrix
		- Differentiation and proliferation (DFC labeled with Brdu): Brdu-positive cells density was higher at PDL > Alveolar Bone > Dentin-Pulp complex and cementum	
		- Identification of tooth roots – Immunohistochemistry for DMP1, DSP, Col-I: Positive for DMP1 and DSP indicating odontogenic potential; Col-I Positive too	
	Conclusion: DFCs may have the ability to differentiate into pulp-dentin complex cells similar to odontoblasts in vivo. The microenvironment of the alveolar fossa in combination with TDM was found to be as very inductive for tooth root construction.		

<table>
<tr><td rowspan="3">Yang (2012)</td><td rowspan="2">DPSCs
-
Chitosan/collagen scaffolds (freeze-drying process) charged with BMP-7 DNA</td><td>In Vitro – Days 1, 4, 8, 16, 24</td></tr>
<tr><td>Cell Proliferation (DNA content); ALP activity (405nm – ELISA); Calcium content
Gene Expression (Real Time PCR) – OC, BSP, DSPP, DMP-1</td></tr>
<tr><td>In Vivo 4 and 8 wks. Evaluation – Frozen sections of scaffolds and background tissue</td></tr>
<tr><td colspan="3">Histologic Evaluation (H&E)
Immunohistochemistry (Rabbit anti-human DSPP and DMP-1)</td></tr>
<tr><td colspan="4">Conclusion:
- BMP-7 gene-activated chitosan/collagen scaffolds were better for cell viability and proliferation than regular scaffolds
- These gene-activated scaffolds supported the cell proliferation and promoted odontogenic differentiation of DPSCs both in vitro and in vivo.
- This study demonstrated the potential of chitosan/collagen scaffold combined BMP-7 gene as a good substrate candidate in dental tissue engineering.</td></tr>
<tr><td rowspan="8">Zhang (2012)</td><td rowspan="8">Rat DPSC
-
Demineralized Dentin Matrix (DDM); Ceramic Bovine Bone (CBB); small intestinal submucosa (SIS); PLGA 85:15; Collagen–Chondroitin Sulfate–Hyaluronic Acid (Co-CS-HA)

Porogen: 200–280 µm</td><td colspan="2">In Vitro Analysis</td></tr>
<tr><td colspan="2">Colony-forming assay (10³ cells): forming colony cells/ spindle-shaped
Differentiation ability of DPSCs (Adipogenic and Osteogenic medium): Positive for both
RT-PCR (DPSC in Osteogenic medium) – DSPP; DMP-1; β-actin: DSPP and DMP1 expression shown the odontogenic potential</td></tr>
<tr><td>Cell-Seeded Scaffolds</td><td>Cell Cytotoxicity: Negative for all of evaluated Scaffolds
Cell Proliferation – MTS assay 3 and 7 days: DPSCs seeded on 20mm³ scaffolds displayed higher proliferation after 3 and 7 days of cultivation. DDM and SIS > CBB > PLGA and Co-CS-HA
ALP: Positive for all. DDM and CBB > SIS > PLGA and Co-CS-HA
Flow Cytometry (20mm³ Scaffolds)
SEM
qRT-PCR (BSP, OCN, DSPP, DMP1): Cells seeded into five scaffolds were positive for all genes. DMP1 was enhanced in DPSC cultured over DDM and CBB
Western Blot (ERK1/2, JNK, p38, P-ERK1/2, P-JNK, P-p38): ERK1/2 and p38 were higher in DPSCs cultured on DDM and CBB</td></tr>
<tr><td colspan="2">Inhibition of the ERK1/2 and p38 pathways resulted in lower levels of ALP activities and mRNA expression of DSPP and DMP1</td></tr>
<tr><td colspan="2">In Vivo Analysis</td></tr>
<tr><td colspan="2">Histological Analysis: DPSCs combined with different scaffolds generated a large amount of dentin-like tissues. DDM and CBB > SIS > PLGA and Co-CS-HA
qRT-PCR: Tissues formed by Rat DPSC expressed DSPP and DMP1. Higher expression by tissues growth in DDM and CBB</td></tr>
<tr><td colspan="3">Conclusion: The five kinds of scaffolds supported the attachment, growth, and differentiation of DPSCs. Natural scaffolds (DDM, CBB, and SIS) had greater osteogenic ability than the synthetic and composite scaffolds (PLGA and Co–CS–HA).</td></tr>
</table>

<table>
<tr><td rowspan="6">Chen (2012)</td><td rowspan="5">DPSC (41) – 19-23 years of age human – SCAPs for alternative in vivo experiment
-
HA/TCP
-
Platelet Lysate (PL) as substitute of FBS</td><td colspan="2">In Vitro Analysis</td></tr>
<tr><td colspan="2">Immunocytochemical staining (STRO-1 and Dsp): Positive
Flow Cytometry (STRO-1-, CD29-, CD34-, CD44-, CD45-, CD90-, CD105- and CD146): Negative for CD34 and CD45
Odontogenic Induction: Positive Alizarin Red and DSP</td></tr>
<tr><td>Cell Culture Media</td><td>Normal Media (N) and Odontogenic Media (M) with or without addition of PL (1%, 5% and 10%)</td></tr>
<tr><td>Culture plates</td><td>Cell Proliferation (1-14 days): after 5 days, a significant enhancement in cell proliferation (1% and 5% PL-containing groups).
ALP: 1% PL/M and 5% PL/M increased ALP activity
PL/M enhances the mineralized differentiation of DPSCs</td></tr>
<tr><td>HA/TCP cell-seeded</td><td>Cell Proliferation: Comparing Control/M and Control/N group with PL treated media, 1% PL/M and 5% PL/M resulted in a higher proliferation rate at day 7 and day 10
ALP: PL increased Alp activity
SEM: cells treated with 5% PL/M and 1% PL/M had fully covered the HA/TCP Surface. After 7 days, the cells treated with 5% PL/M and 1% PL/M reached a stratified confluence and collagen-like structures and abundant calcified ECMs could be observed at the same time (AW)</td></tr>
<tr><td colspan="2">In Vivo Implantation – After the samples of cell-seeded biomaterials were cultured in odontogenic/osteogenic differentiation media supplemented with 5% platelet lysate (PL),1% PL, or without PL for 14 days, the samples were transplanted subcutaneously into the dorsal region of immunodeficient female mice</td></tr>
<tr><td colspan="2">Analysis in the root fragment human (Huang et al., 2010) – 5% PL/M had a continuous layer of odontoblast-like cells lined against the dentin wall (SCAPs > DPSC)</td></tr>
<tr><td colspan="3">Conclusion: The addition of 5% PL to the culture medium results in the greatest induction of ALP activity and odontogenic/Osteogenic differentiation. The authors have concluded that 5% PL was the most effective concentration for proliferation and odontoblastic differentiation of cell cultured in vivo and in vitro onto HA/TCP scaffolds</td></tr>
<tr><td rowspan="3">Kodonas (2012)</td><td rowspan="2">Cryopreserved swine dental pulp stem cells (S-DPSCs)
PLGA (50:50)
Salt (75-150 µm) leaching technique
-
Collagen I sponge (pore size ± 300 µm</td><td colspan="2">In Vivo Analysis – Constructs Transplantation was made using the “Root Implant Model” – Root segment (RS) = 5 to 6mm in length immersed in 3% NaOCl 5 min. RS were filled with PLGA/Col-I+S-DPSCs. Implants were retrieved after 6 and 10 Wks.</td></tr>
<tr><td colspan="2">- Histological Analysis: resorption lacunae with odontoclastic activity at the external root surface; 6 wks: cell proliferating into root canal with signs of scaffold (PLGA an Col-I) degradation. Extracellular matrix deposition in a predentin-like pattern on the canal dentinal walls by cuboidal cells. 10 wks: new organic matrix had been deposited on the canal walls. Presence of odontoblast-like cell in a continuous layer associated with new-deposited matrix.
- Immunohistochemical Examination (DMP1 and BSPII): confirmed the presence of DMP1 and BSP on the sites were odontoblast-like cells were differentiated</td></tr>
<tr><td colspan="3">Conclusion: The interactions of S-DPSCs with the dentin matrix of roots implanted in the jawbone of mini pigs constitute a model to study in vivo organization and differentiation potential of DPSCs.</td></tr>
</table>

Table 4. Strategies of pre conditioning dentin that produced results with potential to be applied clinically in future therapies

Study	Dentin Conditioning
Huang (2006) **Huang (2010)**	*2006*: Dentin discs (*in vitro*) *2010*: Root canals mechanically prepared (opening ~2,5mm) - 17% EDTA for 10 min and - 19% citric acid for 1 min - 5.25% NaOCl for 30 min;
Gotileb (2008)	- Root canal mechanically prepared and constantly irrigated with 6% NaOCl - 17% EDTA for 1 min.
Cordeiro (2008)	Pulp gently removed to maintain the intact pre-dentin
Casagrande (2010)	Tooth Slice/Scaffolds (TS/S) submitted to different conditions prior cell seeding: - TS/S1- 5.25% NaOCl for 5 days at 4°C; TS/S2- 10% EDTA (pH 7.2) for 1 min at 4°C; TS/S3- untreated control TS/S; S4- control PLLA scaffolds without the - tooth slice(TS)
Sakai (2010) **Demarco (2010)**	- Without mechanical preparation. - 10% EDTA for 1 min.
Galler (2011)	- Group A: 10 minutes in 5.25% NaOCl; - Group B: 10 min in 5.25% NaOCl + 2 min 1% EDTA
Galler (2012)	Dentin cylinders (inner diameter: 1mm) - Canal enlarged by a diamond burr - 5% NaOCl for 10 min - 17% EDTA for 2min
Guo 2012	Treated Dentin Matrix applied as an inductive microenvironment/scaffold for the pulp-dentin complex inserted alveolar fossa

Artigo 2: Influence of scaffold pore size on Dental Pulp Stem Cells proliferation and differentiation. Formatado segundo as normas do periódico International Endodontic Journa (Qualis: A2– JCR: 2.193)

Influence of scaffold pore size on Dental Pulp Stem Cells proliferation and differentiation

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Influence of scaffold pore size on Dental Pulp Stem Cells proliferation and differentiation

Abstract

Aim: was to evaluate the influence of the pore size on the proliferation and differentiation of Dental Pulp Stem Cells (DPSCs) *in vitro*.

Methodology: In order to obtain two different pore sizes (150-250µm and 251-450µm), sodium chloride was sieved and used as the porogen-inducer. Tooth slices (1-mm thickness) were obtained from recently extracted third molars and after pulp tissue removal, scaffolds with both porogen inducer sizes were prepared using PLLA *(Poly-L-lactic acid) inside the pulp chamber.* DPSCs (1×10^5 cells) were seeded in the scaffolds with different porosities, in 24-well plates with specific medium. The cell proliferation was evaluated using the WST1 assay at 3, 7, 14 and 21 days intervals. Also, after 21 days of culture, the RNA of seeded cells was extracted using Trizol and RT-PCR technique was used to assess the differentiation of the DPSCs in odontoblasts, using putative odontoblast markers (DSPP, DMP1 and MEPE). RNA from fresh odontoblasts was used as a control.

Results: Cell proliferation rate was similar in both scaffolds except for the 14 days period, when the cells seeded in the scaffolds with larger porosities showed higher proliferation ($p<0.05$). After 21 days DPSCs seeded into the dentin slices expressed the differentiation odontoblastic markers, independently of the pore sizes.

Conclusion: The two different pore sizes tested allowed the DPSCs proliferation and differentiation.

1. Introduction

Dental pulp is a loose mesenchymal tissue characterized by its particular location, which is almost entirely enclosed in a mineralized tissue. Damage to dental pulp can induce various types of inflammatory responses (Bjorndal & Mjor 2001). Pulp Inflammation usually involves complex vascular, lymphatic, and local tissue reactions. In pathological conditions, such as mild carious dentine lesions, odontoblastic activity is stimulated to elaborate reactionary dentine. However, in cases of severe lesions or deep cavity preparations odontoblasts may be lost and dental pulp may undergo necrosis (Heyeraas et al. 2001). Traditionally, when necrotic pulp needs to be removed a conventional root therapy is performed. Despite the good results observed following endodontic therapy (Friedman & Mor 2004)in some situations this treatment option could not be the first choice.In young patients following incisors trauma, causing pulp necrosis, endodontic therapy may weaken the root with large root channel favoring the occurrence of root fracture in case of a new trauma. In this case, tissue engineer approach to develop a new vital pulp tissue seems to be of remarkable importance (Nör 2006).

The three key elements for tissue engineering are: responsive cells, morphogens and scaffolds (Nör 2006). Scaffolds are three-dimensional structures used to support and guide the in-growth of cells, forming the template for cell colonization, proliferation, and providing different sets of physiological signals to the developing tissue (Zhu 2010). Scaffolds should mimic the physical and biological function of the native extracellular matrix (ECM),which provides a physical substrate for cells and it has also specific bioactive molecules that control cellular behavior (Choi et al. 2010; Choi et al. 2012).

Scaffold's pore structure and size are regarded as important aspects to be considered, since they have shown to affect cell binding, migration, depth of cellular in growth, cell morphology and phenotypic expression (Lawrence & Madihally 2008; Demarco et al. 2010; Murphy & O'Brien 2010; Demarco et al. 2011; Santana et al. 2012). Scaffold pores must be interconnected to allow cell growth, migration and nutrition flow. When pore size is small it can provide a strong cell attachment;

however, cell migration can be compromised, resulting in the formation of a cellular capsule around the edges of the scaffold (Lawrence & Madihally 2008). This could limit diffusion of nutrients and removal of waste resulting in necrotic regions within the construct, as well as compromising the interaction between reconstructed structure and native surrounding tissue. On the other hand, if pore size is larger they can allow effective nutrient supply, gas diffusion and metabolic residue removal, but can also lead to low cell attachment and intracellular signaling (Murphy et al. 2010; Murphy & O'Brien 2010).

Studies have demonstrated that the most favorable pore size range depends on the material and cell type (Zeltinger et al. 2001; Salem et al. 2002; Lawrence & Madihally 2008). Salem et al. (Salem et al. 2002) has shown that endothelial cells bind to silicon nitride scaffold with pores preferentially smaller than 80 µm, while fibroblast binds to pores > 90 µm (Salem et al. 2002). In PLLA scaffolds, vascular smooth muscle cells bind to a smaller pore range size of 63-150 µm, while fibroblast bind to a wider range - 38-150 µm (Zeltinger et al. 2001). In addition, the minimum pore size required for bone tissue engineering is generally ~100 µm, where larger pores (100-150 and 150-200 µm) have shown substantial bone ingrowth, an smaller pores (75-100 µm) results in ingrowth of unmineralized osteoid tissue (Karageorgiou & Kaplan 2005; Murphy et al. 2010; Murphy & O'Brien 2010). In relation to Dental Pulp Stem Cells (DPSC), Demarco et al. (2010) verified that these cells were able to proliferate in a PLLA scaffolds with a pore size ranging 250-425 µm, prepared in a tooth slice model. Besides, DPSC exhibiting after 14 days gene expression indicative of differentiation towards an odontoblast-like cells phenotype.

This study was designed to test the hypothesis that the size of the scaffold pore size could influence the proliferation and differentiation of Dental Pulp Stem Cells (DPSCs).

2. Materials and methods

Chemicals: Cell culture medium and reagents were supplied by Invitrogen (Grand Island, NY, USA). All the other reagents were obtained from Sigma Aldrich Chemical Co (St. Louis, MO, USA), except quoted and phosphate buffered saline which was obtained from Mediatech, Inc. (Herndon, VA, USA). Poly-L-lactic acid (Boehringer Ingelheim, Germany)

Cells: Dental Pulp Stem Cells (DPSC), provided by Dr. Songtao Shi from the Dental Biology Unit, Craniofacial Skeletal Diseases Branch, NIH Bethesda, MD, were cultivated in Dulbecco's Modified Eagle Medium (DMEM) low glucose containing 10% fetal bovine serum and 1% penicillin/streptomycin solution and incubated at 37^{o}C in 5% CO_2. During the experiments cells from passage 4-6th were used.

Porogen-inducer production and scaffold preparation: Sodium chloride (salt) was sieved using metallic sieves producing two particle sizes: 150-250 µm and 251-425µm (Demarco et al. 2010). After sieving, salt was stored in Petri dishes until test. Tooth slices (1-mm thickness) were obtained from recently extracted third molars as previous reported (Demarco et al., 2010) the dentin was conditioned for 1 minute with EDTA and washed again with PBS.

Cell seeding: DPSCs at the subconfluent stage (80%) were detached with 0.25% trypsin-EDTA (Gibco, Invitrogen, Grand Island, NY, USA) and a total of 1 x 10^5, in a 20µl cell suspension (DMEM), were seeded inside each PLLA scaffold, and placed in a 24 well plate. Immediately after seeding, samples were placed in incubator (37^{o}C in 5% CO_2) for 1 hour to allow initial cell attachment. Then, 500µl DMEM low glucose was added in each well, being the medium changed every other day.

Cell proliferation: Cell proliferation in the scaffolds was checked after 3, 7, 14, and 21 days, using the WST1 dye as described previously (Nedel et al. 2011). Briefly, at each time point, 20µl WST1 was added to 200µl of new DMEM medium and incubated for 1 hour to develop the reaction. Then, 100µl was removed for each well and placed into a 96-well plate and the optical density was determined in an ELISA multiplate reader (TECAN, Genius) using a wavelength filter of 450 nm. Data were submitted to statistical analysis using two–way ANOVA followed by Tukey test using Sigmastat

2.0 software (SSPS, Chicago, IL, USA) and the significant level was determined at $p<0.05$. For each condition/time triplicates were performed and the experiments were repeated at least three times.

Gene expression (RT-PCR): Similarly, DPSC cells were seeded in tooth slices-scaffolds prepared with both pore sizes. After 21 days in culture, three scaffolds per condition were pooled and 1ml of Trizol® was added, following a standard protocol to extract the RNA. Total RNA (0.2µg) was used in a reverse transcriptase polymerase chain reaction (RT-PCR). The human-specific sense and antisense primers were designed according to published cDNA sequences of GenBank (Table 1). From freshly extracted third molars RNA (control) was extracted using the Trizol protocol previously described (Demarco et al., 2010). Three independent experiments were performed to verify the reproducibility of the results.

3. Results

The two sizes of porogens evaluated on this study produced different scaffolds, as observed in the Figure 1, by optical microscopy.

Cell proliferation: Both pore sizes evaluated produced similar cell proliferation in the different days tested. However, it can be observed that in the fourteenth day the cell proliferation was higher in the scaffolds with larger porosities (Figure 2). The cells in both scaffolds had a continuous growth up to the 21 days, when a slight decrease was observed, indicating that cells were differentiation and also possibly reaching confluence.

Cell gene expression: DPSCs seeded in the tooth slices scaffolds with both pore sizes exhibited after 21 days the expression of all putative odontoblast markers (DSPP, DMP1, and MEPE), similarly to control. House-keeping gene was also expressed by control and experimental groups (Figure 3).

4. Discussion

Scaffold mean pore size is critical for cell growth and migration (O'Brien et al. 2005; El-Backly et al. 2008; Murphy et al. 2010) and there is an optimum mean pore size to allow cell viability. Here, we have chosen to evaluate the PLLA scaffold pore size considering the lack of information about this specific topic in the dental tissue engineering.

We have used the Tooth-Slice Scaffold (TS/S) model, which has demonstrated its efficiency to allow proliferation and differentiation of stem cells from dental tissues (Sakai et al. 2011). Demarco et al (Demarco et al. 2010) showed that the scaffold characteristics could be tested in this model, comparing two scaffolds with different porous formats using *in vitro* and *in vivo* tests. In that study, the authors were able to show that both porogen inducers provided an adequate and similar environment for cell proliferation and differentiation, expressing the three putative odontoblastic markers. Thus, we tested whether the same pore size applied in the previous study and a smaller one could effectively affect the DPSC proliferation and differentiation since it was previously reported that smaller porous sizes increases the specific surface area for cell attachment (El-Backly et al. 2008). Besides, the fraction of cells attached to the scaffold decreases with increasing mean pore size and increases linearly with specific surface area, consistent with the increase in ligand binding site density at short-time periods (O'Brien et al. 2005).

WST-1 test have been showing to allow the determination of cell density of DPSCs in different time periods with a simpler methodology (Nedel et al. 2011). Here we observed an increased cell proliferation over time until cell confluence or differentiation had happened (Casagrande et al. 2010). WST-1 showed difference in cell proliferation rate just at day 14, when the cells seeded in the larger porosities (251-425 μm) showed a higher proliferation rate than those seeded in the scaffolds with smaller porosities (150-250μm). These records endorse the affirmative, which says that the scaffolds should be sufficiently permeable to facilitate cell growth, migration and nutrition (Lawrence & Madihally 2008). Based on that, we can speculate that higher pore size allowed a faster DPSC migration and proliferation until them reached the confluence faster than cells

seeded onto scaffolds with smaller pores. In fact, Murphy et al., (Murphy et al. 2010) comparing a range of scaffolds, with different porosities, showed that higher pore size improved cellular adhesion and infiltration into scaffolds up to 7 days post-seeding. However, those results were obtained in a series of collagen–glycosaminoglycan (CG) scaffolds; cells can discriminate subtle changes in the ECM that may affect their behavior; consequently we should be cautious when comparing such results.

In the meantime, we observed a consistent slowdown in total cell proliferation at day 21 into scaffolds with large pore size, being it similar to observed in the scaffolds with smaller pore sizes. In theory, we can work with two hypotheses: 1) cells had reached confluence or have formed tissues that had achieved homeostasis; 2) the high cell densities, provided by larger mean pores, inhibit the proliferation by cell-cell contact and it was accentuated by crowding the spaces, which induces a morphologic alteration in cells and could induce cell death (Lanosa & Colombo 2008). It is interesting to point out that scaffold pore size affects other stem cells' behavior behind cell proliferation. In this context deep analyses should be carried out because other variables can be responsible for cell density decrease. The migration ability decreases as scaffolds pore size increases (Harley et al. 2008) in CG scaffolds. Moreover, adherent cell has contractile properties, which induces buckling a strut within the scaffold (Corin & Gibson 2010) and probably influences DPSC behavior.

In addition to DPSC proliferation rates, we evaluated the influence of pore size in DPSC differentiation. We could observe after 21 days the stem cells differentiation towards an odontoblast phenotype when seeded in TS/S, independently of the size of the porosities. DPSCs seeded in the tooth slice scaffolds showed the expression of the three putative odontoblast markers after 21 days, as previously reported in other studies of our lab (Casagrande et al. 2010; Demarco et al. 2010; Sakai et al. 2010).

To assess the odontoblastic differentiation we used DSPP, DMP-1 and MEPE gene expression. DSPP and DMP-1 are expressed by differentiating odontoblasts (D'Souza et al. 1997) and the presence of DMP-1 and DSPP in functional odontoblasts prior to mineralization is consistent with the hypothesis that both DMP-1 and DSPP play a role in the mineralization of dentin (D'Souza et al.

1997). DMP-1 is a preodontoblastic protein, which can bind to calcium due its acidic nature, thereby initiating the hydroxyapatite nucleation processes (Linde & Goldberg 1993). Even though DSPP and DMP-1 are expressed in other body tissues (Qin et al. 2002; Baba et al. 2004; Qin et al. 2007), their expression can be considered as an indicator of odontoblastic differentiation (Goldberg & Smith 2004). Although, some controversy in relation to its temporal expression (Liu et al. 2005; Demarco et al. 2010), MEPE has been used to indicate differentiation of stem cells towards odontoblastic phenotype (Conde et al. 2012). After dentin mineralization, bioactive molecules, including growth factors and non-collagenous proteins - DSPP, DMP-1 and MEPE - are fossilized into dentin matrix, may be released from the dentin matrix to allow reparative dentin formation (Graham et al. 2006; Tomson et al. 2007). Casagrande et al. (2010) demonstrated that morphogenic signals released from dentin were sufficient to stimulate the SHED differentiation to odontoblast-like cells. Similarly, Sakai et al., (Sakai et al. 2010) showed that SHED cells were not only differentiated when seeded in tooth slice scaffolds, but these cells were also functional odontoblasts produced new dentin. At this study we could observe also, the odontoblast markers expression by stem cell after they seeding and growing in a TS/S model and probably, like in the studies above mentioned its differentiation was induced by growth factor released from the dentin disc.

Conclusion

Here was possible to observe that the two mean pore sizes evaluated here provided a favorable environment for DPSC proliferation and differentiation

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Figure 1. Optical microscopy showing the two different scaffolds produced (40x magnification). A) Minor salt particles used as porogen (150-250 µm); B) Major salt particles used as porogen (251-425µm)

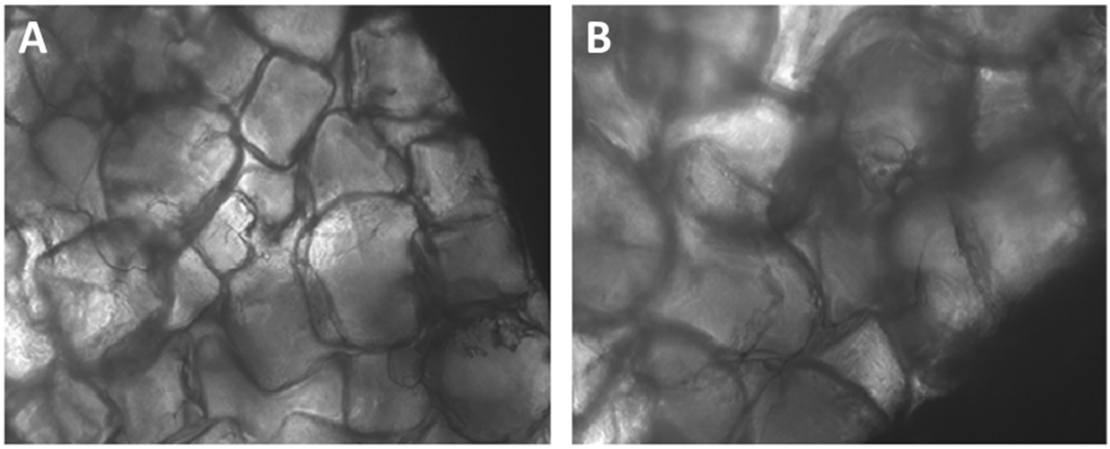

Figure 2. WST-1 analysis demonstrating the cell proliferation rates on the two scaffolds produced. Day 14 (P<0,05).

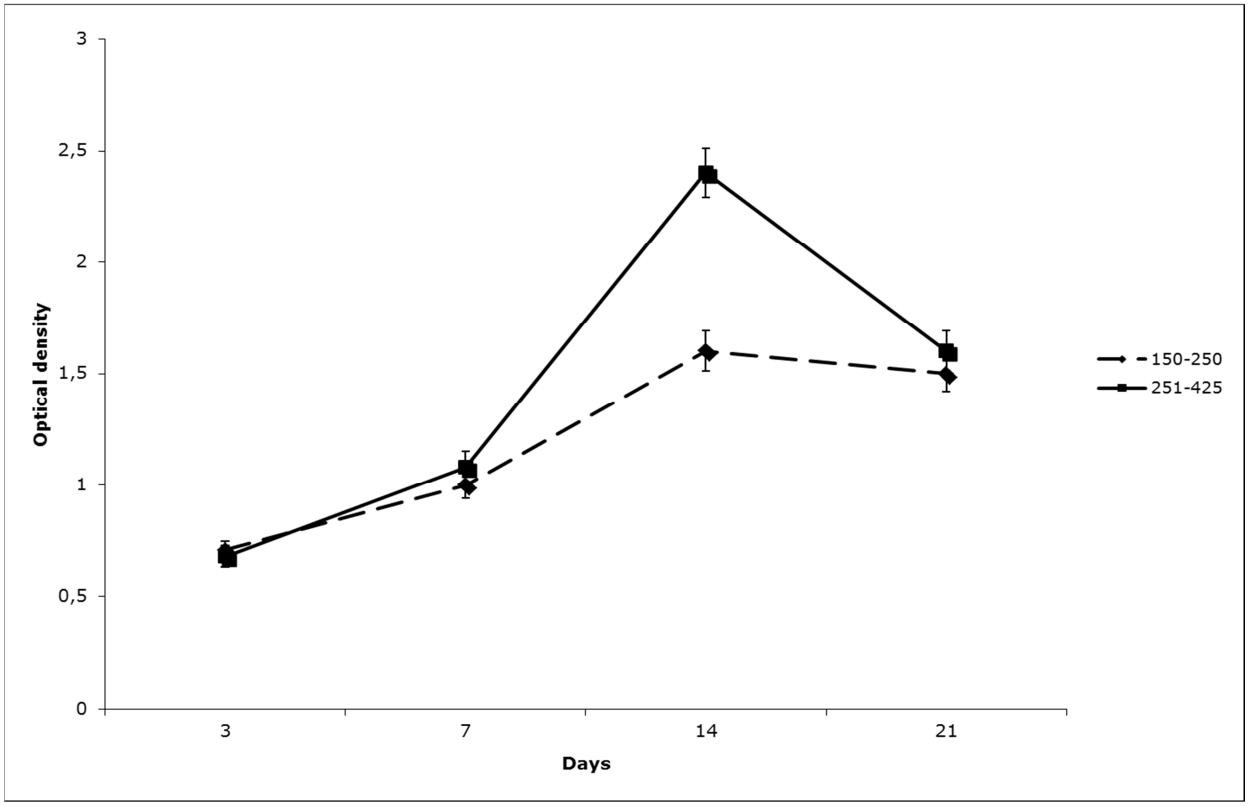

Figure 3. RT-PCR. DPSC were able to express the three odontoblast markers after have been seeded fro 21 days into both scaffolds assessed. OD = Odontoblastic RNA; S< = Scaffolds with minor pore size; S> = Scaffolds with larger pore size. GAPDH: Housekeeping gene

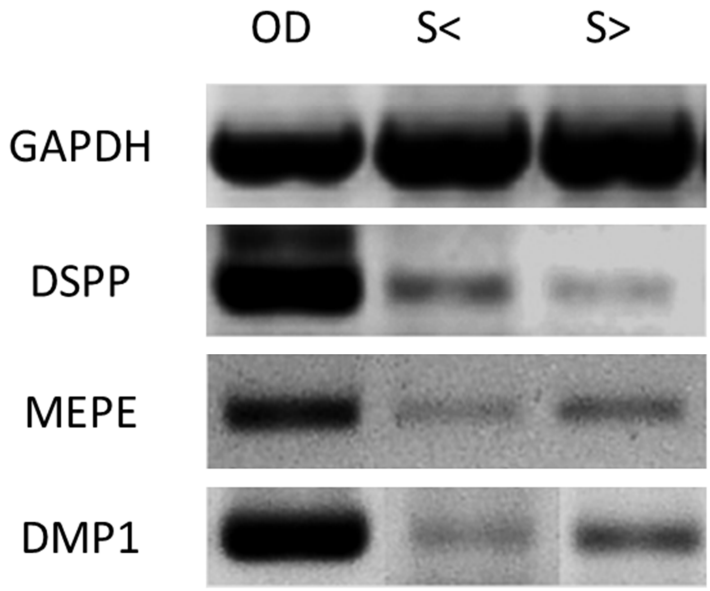

Table 1. Primer sequences and annealing temperatures used for gene expression analyses

Primers Sequence used for gene expression analyses		
	Primer Sequence (Genbank)	Product Size
GAPDH	Forward *5' GACCCCTTCATTGACCTCAACT 3'* Reverse *5' CACCACCTTCTTGATGTCATC 3*	683 bp
DSPP	Forward *5' GACCCCTTCATTGACCTCAACT 3'* Reverse *5' TGCCATTTGCTGTGATGTTT 3'*	181 bp
DMP1	Forward 5' CAGGAGCACAGGAAAAGGAG 3' Reverse *5' CTGGTGGTATCTTGGGCACT 3'*	213 bp
MEPE	Forward 5' GCAAAAGCACCCATCGTATT 3' Reverse 5' CTGCCCTCTACAAGGCTGAC 3'	385 bp

6. Conclusões

i. Ainda não existe um consenso sobre o material ideal para a fabricação de *sacffolds* na engenharia da tecidual da polpa dental. Entretanto, ficou claro que uma ampla gama de materiais é capaz de estabelecer uma interface apropriada entre as células-tronco e os *scaffolds* utilizados. Neste contexto aprincipalestratégia empregada para aprimorar a interação células/scaffolds é a incorporação de moléculas bioativas (BMPs-2, 4 e 7, o TGF-β1, DMP1) na estrutura das matrizes, para tornar a composição química do material, mais semelhante à MEC.

ii. *Scaffolds* com dimensões nanométricas parecem mais promissores no processo transitório de pesquisa laboratorial para ensaios clínicos. Neste contexto, os Polipeptideos Anfífilos demonstraram um grande potencial de aplicalidade devido a sua facilidade de manipulação, biocompatibilidade e injetabilidade (uma de suas propriedades mais atratiivas).A distribuição e tamanho dos poros de um *scaffold*, são determinantes para a fixação, proliferação e diferenciação das céluas com eles implantadas.

iii. Os sinais do ambiente, no qual os *constructs* (Células + *Scaffolds*) são implantados, desempenham um papel crítico na formação de um novo tecido pulpar, sobrepondo, inclusive, as propriedades físico-químicas dos materiais utilizados como *scaffold*.

iv. Para obter a neoformação de tecido similar a polpa dental, o pré-condicionamento da dentinaé de fundamental importância. Alem disso, a habilidade das células-tronco de expressar VEGF e se diferenciar em estruturas endoteliais se mostraram condições indispensáveis para que se supere a “barreira anatomica” do forame apical.

v. Na realização do trabalho experimental, foi confirmada a nossa hipotése de que os scaffolds com menores porosidades seriam adequados para a proliferação e diferenciação odontoblástica das DPSC. Este resultado nos permite vislumbrar a comfecção de scaffolds com diferentes tamanhos de porosidades pra que se possa simular *in vitro* a complexidade da MEC em estudos sobre o comportamento de células-tronco

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