UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
DOUTORADO EM CLÍNICA ODONTOLÓGICA
ODONTOPEDIATRIA

APLICAÇÃO DE PRINCÍPIOS DE ENGENHARIA TECIDUAL NO ESTUDO DA DIFERENCIAÇÃO DE CÉLULAS-TRONCO PULPARES.

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PORTO ALEGRE

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TESE

Aplicação de princípios de engenharia tecidual no estudo da diferenciação de células-tronco pulpares.

LUCIANO CASAGRANDE

PROGRAMA DE DOUTORADO NO PAÍS COM
ESTÁGIO NO EXTERIOR PDEE – CAPES
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Orientação:
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Prof. Fernando Borba de Araujo

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SUMÁRIO

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LISTA DE ABREVIATURAS

ACVR1 – activin-receptor one
ACVR2 – activin-receptor two
cDNA – complementary deoxyribonucleic acid
BMP – bone morphogenetic protein
BMPab – bone morphogenetic protein antibodie
BMPR – bone morphogenetic protein receptor
BMSSC – bone marrow stromal stem cells
DMEM – dulbecco's modified eagle medium
DMP-1 – dentin matrix protein one
DSP – dentin sialoprotein
DPP – dentin phosphoprotein
DPSC – dental pulp stem cells
DSPP – dentin sialophosphoprotein
EDTA – ethylenediaminetetraacetic acid
FBS – fetal bovine serum
FGF – fibroblast growth factor
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
GDF11 – Growth differentiation factor 11
HDMEC – human dermal microvessel endothelial cell
Hh – hedgehog gene
IGF – Insulin-like growth factor
MEPE – matrix extracellular phosphoglycoprotein
MG-63 – human osteosarcoma cell line
mRNA – messenger ribonucleic acid
NaOCl – sodium hypochlorite
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PGA – poly glycolic acid
PLGA – poly lactic-co-glycolic acid
PLLA – poly-L-lactic acid
PSS – poly-L-lactic acid based sponge scaffold
rhBMP – recombinant human bone morphogenetic protein
RNA – ribonucleic acid
RT-PCR – reverse transcriptase polymerase chain reaction
SCID – severe combined immunodeficient
SHED – stem cell from human exfoliated deciduous teeth
TGF_{β} – transforming growth factor-beta
TSS – tooth slice scaffold
UMSCC11A – laryngeal cancer cell line
VEFG – vascular endothelial growth factor
WnT – wingless gene
WO-T – without treatment
WST-1 – proliferation assay

RESUMO

O presente estudo utilizou o modelo fatia-dental/matriz-polimérica para avaliar a influência do tratamento dentinário e das BMPs dentinárias na diferenciação das células-tronco da polpa de dentes decíduos (SHED). Secções transversais (1mm) foram preparadas a partir de terceiros molares humanos extraídos. Matrizes poliméricas a base de ácido poli-L-lático (PLLA) foram criadas no interior da cavidade pulpar das secções dentinárias, tratadas com solução de EDTA a 10%; NaOCl a 5.25%; ou permanecendo sem tratamento. Matrizes poliméricas confeccionadas sem as fatias dentais foram utilizadas como controle. As células ($5x10^4$) foram semeadas nas matrizes e, após 7, 14, 21 e 28 dias de cultura *in vitro*, a expressão de marcadores de diferenciação odontoblástica (DSPP, DMP1 e MEPE) e a proliferação celular (WST-1) foram avaliadas. Células ($5x10^5$) semeadas nas matrizes foram transplantadas em camundongos imunodeficientes e cultivadas *in vivo* por um período de 14 e 28 dias. Para avaliar a atividade das BMPs dentinárias, $5x10^4$ células foram semeadas em matrizes poliméricas com fatia dental e cultivadas na presença de anticorpos anti-BMP-2, -4, ou -7 (2 μg/ml) durante 14 dias. Adicionalmente, $5x10^5$ células foram tratadas com rhBMP-2, -4, ou -7 (100ng/mL) por 24hs. As células cultivadas *in vitro* e *in vivo* alteraram sua expressão genética durante o curso do tempo. DSPP, DMP-1 e MEPE foram expressos por células cultivadas *in vitro* após 14 dias (tratamento com EDTA e dentina sem tratamento) e *in vivo* após 28 dias (EDTA), não sendo detectados nos grupos NaOCl e nas células cultivadas nas matrizes sem fatia dental. A proliferação foi reduzida com a diferenciação celular ($p<0.05$). A utilização de BMP-2/4Ab no meio de cultura exerceu um efeito inibitório na expressão dos marcadores de diferenciação celular, não ocorrendo quando do cultivo das SHED na presença de BMP-7Ab. DSPP, DMP-1 e MEPE foram expressos por células tratadas com rhBMP-2, e DSPP e DMP-1 por células tratadas com rhBMP-4 e -7. Células sem tratamento não expressaram os marcadores. O modelo fatia-dental/matriz-polimérica demonstrou ser adequado para o estudo da diferenciação de células-tronco pulpares, sugerindo que a dentina possa fornecer um microambiente favorável para a diferenciação de celular. As proteínas ósseas morfogenéticas dentinárias BMP-2 e BMP-4 parecem exercer um papel relevante nesse processo.

Palavras-chave: engenharia de tecidos; células-tronco da polpa dental; matrizes poliméricas; dentina; proteínas ósseas morfogenéticas (BMP); proliferação; diferenciação.

ABSTRACT

The effect of dentin pre-treatments and dentin-derived BMPs on SHED differentiation was tested using the Tooth-Slice Scaffold model (TSS). Dentin slices (1mm thickness) were prepared from extracted human third molars. Biodegradable PLLA scaffolds were prepared inside the pulp chamber of the tooth-slices, treated alternatively with a 5.25% NaOCl or 10% EDTA solution, or remaining untreated (WO-T). PLLA sponge scaffolds with no tooth-slice (PSS) were used as control. SHED ($5x10^4$) were seeded in TSS and PSS and after 7, 14, 21 and 28 days in culture, RT-PCR (DSPP, DMP1 and MEPE) and WST-1 proliferation assay were performed. Additionally, cells ($5x10^5$) were seeded in TSS and PSS and transplanted into SCID mice (14 and 28 days). To verify the dentin-derived BMPs bioactivity, SHED ($5x10^4$) were cultured in TSS in the presence of anti-human BMP-2, -4, and -7 antibodies for 14 days. Besides, cells in culture were treated with rhBMP-2; -4; or -7 for 24 hours. After *in vitro* and *in vivo* time course, SHED altered their genetic expression. The cells cultured *in vitro* in the TSS (EDTA or WO-T) expressed the differentiation markers after 14 days and maintained expression thereafter. Cell proliferation rate was reduced following the differentiation ($p<0.05$). Cells transplanted *in vivo* expressed DSPP, DMP-1 and MEPE after 28 days (EDTA). No transcripts were found in tooth-slices treated with NaOCl or in PSS groups. BMP-2/4Ab prevented the differentiation process and no inhibitory effect was detected for BMP-7Ab. After 24 hours, expression of DSPP, DMP-1 and MEPE was found for rhBMP-2, and DSPP and DMP-1 for rhBMP-4 and rhBMP-7 treated SHED, but not for untreated cells. The tooth slice scaffold model suggests that dentin can provide the environment for SHED differentiation and dentin-derived morphogenic signals BMP-2 and BMP-4 play an important role in this process.

Keywords: tissue engineering; stem cell from human deciduous teeth (SHED); scaffolds; dentin; bone morphogenetic protein (BMP); proliferation; differentiation.

1. INTRODUÇÃO

1.1 Engenharia tecidual

O edentulismo continua sendo um grave problema em nosso país. Segundo os resultados de um estudo que avaliou as condições de saúde bucal da população brasileira, a necessidade de algum tipo de prótese começa a surgir a partir da faixa etária de 15 a 19 anos de idade (Brasil, 2004).

Avanços na área de biologia molecular e celular têm contribuído para o desenvolvimento de técnicas de reparação ou, até mesmo, de regeneração de órgãos e tecidos injuriados por doenças, traumas e deformidades congênitas. Langer e Vacanti (1993) foram os pioneiros a descrever o conceito de engenharia tecidual como sendo um campo interdisciplinar que aplica princípios da engenharia, biologia e ciências clínicas para o desenvolvimento de substitutos biológicos que possam manter, restaurar, ou melhorar a função de órgãos e tecidos. Essa nova ciência está alicerçada sobre três pilares: células, matrizes biocompatíveis e moléculas bioativas responsáveis pelos sinais morfogênicos.

Substitutos biológicos de pele em crianças que sofreram queimaduras severas (HOHLFELD *et al*., 2005), bem como a construção de tecido ósseo em pacientes com perda óssea severa (WARNKE *et al*., 2004) são exemplos de estratégias baseadas na engenharia tecidual que têm sido utilizadas em humanos.

Nos últimos anos, a odontologia passou a explorar o potencial da engenharia tecidual na reparação e regeneração de estruturas dentais. A

descoberta de células-tronco no órgão pulpar de dentes permanentes (GRONTHOS *et al*., 2000) e até mesmo em dentes decíduos (MIURA *et al*., 2003), associada à possibilidade de utilização terapêutica, estimula as especulações para o desenvolvimento de uma "terceira dentição", o que possibilitaria o restabelecimento da função mastigatória e estética com terapias mais biológicas.

1.1.1 Células-tronco pulpares

Todos os órgãos e tecidos do organismo são originados a partir de células-tronco (SMITH, 2001). As células-tronco são comumente definidas como células clonogênicas não especializadas que possuem habilidade de se dividir continuamente e gerar células progenitoras que se diferenciam nas mais diversas linhagens celulares (RAO, 2004). De acordo com a origem, as células-tronco também podem ser classificadas como células embrionárias/fetais ou células-tronco adultas/pós-natais (FORTIER, 2005).

A principal diferença entre as células-tronco embrionárias e pós-natais está na diferença de plasticidade, isto é, no potencial que elas possuem em originar diferentes linhagens celulares especializadas (MARTIN-RENDON e WATT, 2003). O grande potencial de plasticidade faz das células-tronco embrionárias uma alternativa promissora para o desenvolvimento de novas terapias celulares. Porém, a utilização dessa fonte celular altamente indiferenciada é controversa e cercada por questões éticas e legais, o que reduz a sua atratividade e disponibilidade (GARDNER, 2002). Dessa forma, várias

pesquisas estão focadas no desenvolvimento de terapias utilizando células-tronco pós-natais, obtidas do próprio paciente (autógenas) ou através de técnicas alogênicas ou xenogênicas.

As células-tronco pós-natais são células indiferenciadas que caracteristicamente geram tipos celulares compatíveis com o tecido no qual elas residem. Possuem a capacidade de auto-renovação e sua função principal é manter e reparar os tecidos no qual se encontram. Porém, podem também formar tipos celulares especializados de outros tecidos onde forem transplantadas, reprogramando-se de acordo com o novo ambiente, o que é conhecido como trans-diferenciação (BJORSON *et al*., 1999; ANDERSON, GAGE e WEISSMAN, 2001). Sugere-se que a capacidade e o potencial para as células-tronco adultas diferenciarem em um amplo espectro fenotípico é determinado pela interação delas com células residentes nos tecidos, assim como com os fatores de crescimento/diferenciação (WAGERS e WEISSMAN, 2004; VASSILOPOULOS e RUSSELL, 2003).

A aplicação de princípios de terapia celular baseados em células-tronco pós-natais não é muito recente. Em 1968, o primeiro transplante alogênico de medula óssea foi utilizado com sucesso no tratamento de imunodeficiência severa (KENNY e HITZIG, 1979). Desde a década de 1970, transplantes de medula óssea têm sido utilizados freqüentemente para o tratamento de leucemia, linfoma e anemias, além de desordens genéticas (BARRETT e McCARTHY, 1990).

Atualmente, as células-tronco pós-natais têm sido isoladas dos mais

variados órgãos e tecidos como: medula óssea, tecido nervoso, pele, retina, folículos pilosos, intestino, pâncreas, e até mesmo da polpa dental humana (HARADA *et al*., 1999; FUCHS e SEGRE, 2000; GRONTHOS *et al*., 2000; GRONTHOS *et al*., 2002; MIURA *et al*., 2003).

O interesse pelo desenvolvimento de terapias celulares tem aumentado a busca por células-tronco adultas de alto potencial de proliferação e de diferenciação, provenientes de fontes acessíveis. Utilizando técnicas que envolvem a digestão do tecido pulpar através de um processo enzimático, populações de células-tronco pulpares foram primeiramente encontradas na polpa dental de dentes permanentes (DPSC – Dental Pulp Stem Cells - GRONTHOS *et al*., 2000) e, mais tarde, em polpa de dentes decíduos (SHED – Stem Cells from Human Exfoliated Deciduous Teeth - MIURA *et al*., 2003). A análise do comportamento celular revelou características básicas compartilhadas por outras populações de células-tronco pós-natais: auto-renovação e alta capacidade de proliferação e diferenciação (GRONTHOS *et al*., 2002).

Um estudo utilizando marcadores de microvasculatura sugeriu que as células-tronco pulpares estão intimamente associadas com os vasos sanguíneos do tecido pulpar, especialmente pericitos e células musculares lisas. As células-tronco pulpares expressam o fator CD146, alpha-actina da musculatura lisa e proteína 3G5 (SHI e GRONTHOS, 2003).

Em estudos de caracterização fenotípica, células-tronco de medula óssea (Bone Marrow Stromal Stem Cells – BMSC) e DPSC apresentaram algumas

características semelhantes à fibroblastos, células endoteliais, musculatura lisa e osteoblastos. As BMSSC e DPSC expressaram marcadores ósseos, como a sialoproteína, fosfatase alcalina, colágeno tipo I e osteocalcina (GRONTHOS, et al., 2000; SHI, ROBEY e GRONTHOS, 2001). De acordo com Liu *et al*. (2005), células-tronco da polpa dental apresentam o fenótipo fibroblástico, quando se refere ao padrão de proliferação e atividade mineralizadora.

A comparação do potencial de proliferação e diferenciação entre BMSC e DPSC demonstrou que a eficiência na formação de colônias *in vitro* foi maior para as células-tronco de origem pulpar (GRONTHOS *et al*., 2000). De acordo com o estudo, as DPSC possuem a capacidade de regenerar o complexo dentino-pulpar. Após o transplante *in vivo*, as células foram capazes de formar dentina ectópica e tecido pulpar adjacente, representando uma nova população de células-tronco pós-natais com alto potencial proliferativo, capacidade de auto-renovação e diferenciação em múltiplas linhagens.

Em comparação com BMSC e DPSC, as SHED demonstraram um maior potencial de proliferação e capacidade osteoindutora *in vivo,* porém falharam em reconstituir um complexo dentino-pulpar organizado, talvez por terem características mais imaturas que outras populações de células-tronco pós-natais (MIURA *et al*., 2003).

1.1.2 Matrizes biocompatíveis

Para que a engenharia tecidual se constitua em uma opção terapêutica viável na regeneração do complexo dentino-pulpar, são necessárias estruturas

tri-dimensionais de suporte celular e que permitam a re-vascularização do tecido pulpar (NAKASHIMA, 2005).

Os *scaffolds* são estruturas tri-dimensionais que proporcionam um microambiente capaz de permitir a adesão e migração celular. Esses devem apresentar características físicas, químicas e biológicas favoráveis ao crescimento e diferenciação celular, além de microporosidades para permitir a conectividade entre o enxerto e o tecido adjacente, facilitando o transporte de nutrientes e a eliminação dos produtos do metabolismo celular (SACHLOS e CZERNUSKA, 2003; NAKASHIMA e AKAMINE, 2005).

Dependendo da finalidade de aplicação, os *scaffolds* podem ser naturais ou sintéticos, biodegradáveis ou permanentes. *Scaffolds* contendo componentes inorgânicos, tais como hidroxiapatita e fosfato de cálcio, são usualmente empregados na neo-formação óssea guiada (JADLOWIEC, CELIL e HOLLINGER, 2003). Polímeros naturais, a base de colágeno e glicosaminoglicano, oferecem boa bioatividade e biocompatibilidade.

Polímeros sintéticos permitem a manipulação das propriedades físico-químicas como o índice de degradação, tamanho das microporosidades e resistência mecânica. Usualmente, os polímeros sintéticos utilizados na engenharia tecidual são à base de ácido poli-L-lático (poly-L-lactic acid – PLLA), ácido poli-glicólico (poly glycolic acid – PGA), e seus co-polímeros como o ácido poli-lático co-glicólico (poly lactic-co-glycolic acid – PLGA). Essas matrizes poliméricas têm demonstrado bons resultados nas aplicações de engenharia tecidual em função da capacidade de sustentar o crescimento e a diferenciação

celular (TAYLOR *et al*., 1994). Além disso, a taxa de degradação observada é compatível com o índice de formação tecidual, isto significa que as células fabricam sua própria matriz tecidual enquanto o *scaffold* fornece integridade estrutural, até que o tecido neo-formado apresenta-se em condições para auto-sustentação (FREED *et al*., 1994). Matrizes a base de polímeros biodegradáveis (PGA/PLLA) serviram para a engenharia de estruturas dentais com características muito similares à coroa de dentes naturais (YOUNG *et al*., 2002).

1.1.3 Moléculas bioativas do complexo dentino-pulpar

Fatores de crescimento, moléculas bioativas ou fatores morfogenéticos são proteínas que se ligam a receptores celulares induzindo a proliferação/diferenciação celular (WINGARD e DEMETRI, 1999). Atualmente, uma variedade de moléculas bioativas com funções específicas têm sido empregadas na terapia com células-tronco e na engenharia tecidual (RAMOSHEBI *et al*., 2002; VASITA e KATTI, 2006). Diversos fatores de crescimento podem ser utilizados para controlar a atividade de células-tronco, seja aumentando o índice de proliferação, induzindo a diferenciação em outro tipo celular, ou ainda, estimulando as células à sintetizar e secretar matriz mineralizada (MARTIN *et al*., 1998; STEVENS *et al*., 2005).

Os fatores de crescimento exercem um papel fundamental na sinalização de eventos formadores e reparadores do complexo dentino-pulpar. O tecido da polpa dental é reconhecido por sua alta capacidade de reparação (NAKASHIMA e REDDI, 2003; GOLDBERG *et al*., 2003; TZIAFAS, 2004). A vitalidade do

complexo dentino-pulpar após injúria está na dependência da atividade celular e dos processos de sinalização. A formação da barreira mineralizada em locais de exposição pulpar após o capeamento direto é um exemplo clássico do potencial de reparação da polpa dentária (SMITH *et al*., 2000).

A matriz dentinária é considerada como um reservatório de fatores de crescimento, uma vez que TGF_{β} (Transforming Growth Factor), BMP (Bone Morphogenic Protein), FGF (Fibroblast Growth Factor), IGF (Insulin-like Growth Factor) e VEGF (Vascular Endothelial Growth Factor) são incorporados durante o processo de odontogênese e permanecem "fossilizados", porém capazes de estimular resposta tecidual após serem mobilizados (FINKELMAN *et al*., 1990; RUCH, LESOT e BÈGUE-KIRN, 1995). Uma vez liberadas, essas moléculas podem desempenhar papel chave na sinalização de diversos eventos, como a formação da dentina terciária e reparo pulpar (TZIAFAS, 1995).

A estrutura tubular confere à dentina uma permeabilidade significativa (PASHLEY *et al*., 2002) de forma que a difusão de produtos da degradação da matriz dentinária, em conseqüência da desmineralização ocasionada por lesão de cárie, agentes condicionadores dentinários, e materiais capeadores, induz uma seqüência de eventos celulares envolvendo o recrutamento de células-tronco, a diferenciação e a ativação da atividade secretória de matriz mineralizada (SMITH *et al*., 1995; MURRAY e SMITH, 2002).

Quatro grupos distintos de fatores de crescimento parecem estar envolvidos com o desenvolvimento crânio-facial (BMPs, FGF, Wnts, Hhs), contudo as proteínas ósseas morfogenéticas (BMP) parecem ser suficientes

para promover a regeneração dos tecidos dentais (NAKASHIMA e REDDI, 2003). Receptores para BMP (BMPR-IA, IB e II) foram expressos por células extraídas da polpa dental humana (GU, SMOKE e RUTHERFORD, 1996). As BMP são membros da superfamília TGF_{β}, originalmente identificadas como proteínas formadoras de tecido ósseo e cartilaginoso, envolvidas na embriogênese e morfogênese de vários órgãos e tecidos, incluindo os dentes (THESLEFF e SHARPE, 1997).

Os fatores de crescimento BMP-2, BMP-4, BMP-6, BMP-7 e Gdf11 estão envolvidos na proliferação e diferenciação de odontoblastos. Estudos demonstraram que a expressão de BMP-2 está aumentada durante o processo terminal de diferenciação de odontoblastos (NAKASHIMA *et al*., 1994; NAKASHIMA e REDDI, 2003), assim como BMP-7 promove a formação de dentina reparadora e mineralização em modelos animais (DECUP *et al*., 2000; GOLDBERG *et al*., 2001).

Proteínas recombinantes (rhBMP) têm demonstrado induzir a reparação/regeneração dentinária em exposições pulpares (NAKASHIMA, 1994; DECUP *et al*., 2000; SIX, LASFARGUES e GOLDBERG, 2002).

Embora se saiba do papel relevante das BMPs na diferenciação de células-tronco bem como na indução reparadora da polpa dental, os fatores dentinários envolvidos nesse processo não foram totalmente compreendidos e carecem de um maior estudo para que no futuro a regeneração do complexo dentino-pulpar seja uma realidade em uma prática clínica baseada em fundamentos biológicos.

2. OBJETIVOS

A transição da dentição decídua para a permanente é um processo fisiológico único onde o desenvolvimento e erupção dos dentes permanentes se dá simultaneamente à reabsorção da raiz dos dentes decíduos. Essa condição torna o dente decíduo um doador em potencial de células indiferenciadas para diferentes propósitos terapêuticos, principalmente no âmbito da regeneração do complexo dentino-pulpar.

Utilizando os princípios da engenharia tecidual, onde células-tronco da polpa de dentes decíduos (SHED) são semeadas dentro de matrizes poliméricas biodegradáveis criadas no interior de fatias dentais, o presente estudo propõe-se em avaliar:

1) o efeito do pré-tratamento dentinário sobre a proliferação (*in vitro*) e diferenciação (*in vitro* e *in vivo*) das SHED utilizando o modelo fatia-dental/matriz-polimérica;

2) a influência das BMPs dentinárias na diferenciação das células-tronco da polpa de dentes decíduos.

3. ARTIGO 1

The effect of dentin pre-treatment on SHED differentiation

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Abstract

The effect of different dentin pre-treatments on the differentiation of stem cells from human exfoliated deciduous teeth (SHED) was evaluated using a Tooth-Slice Scaffold Model (TSS). Dentin tooth-slices (1mm thickness) were prepared from extracted human third molars. Biodegradable highly porous poly-L-latic acid (PLLA) scaffolds were fabricated in the pulp space of the tooth-slices. These scaffolds were untreated (WO-T), treated alternatively with 5.25% sodium hypochlorite solution (NaOCl), or with ethylenediaminetetraacetic acid (EDTA). SHED (5×10^4) were seeded into the scaffolds and cultured *in vitro* in 24-well plates for 7, 14, 21 and 28 days. PLLA sponges scaffolds with no tooth-slice (PSS) were used as controls. RNA was collected with Tryzol® at each time point and reverse transcriptase polimerase chain reaction (RT-PCR) was performed to evaluate the expression of odontoblast differentiation markers (DSPP, DMP1 and MEPE). SHED proliferation, cultured in TSS under different dentin treatments, was quantified using a WST-1 assay at 7, 14, 21 and 28 days. Additionally, SHED (5×10^5) were implanted into SCID mice using the TSS model. RT-PCR demonstrated that SHED cultured *in vitro* altered their genetic expression during the time course. SHED in the TSS treated with EDTA or WO-T expressed differentiation markers after 14 days and maintained the expression up to 28 days. No expression was detected for those cells cultivated in NaOCl or PSS groups. SHED proliferation rate was significantly lower for cells cultivated in untreated TSS and those treated with EDTA, when compared to PSS and TSS NaOCl treated groups after 28 days culture ($p<0.05$). Cells, which were implanted *in vivo* in the TSS EDTA treated group, expressed DSPP, DMP-1 and MEPE after 28 days implantation. Differentiation markers were not found in cells cultured in TSS NaOCl treated or in PSS groups. The TSS model suggests that dentin is necessary to create a favorable environment for SHED differentiation.

Introduction

Recent advances in molecular and cellular biology have led to developments in stem cell research [1]. Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences to the development of biological substitutes that restore, maintain, or improve tissue function or even an entire organ [2]. It uses a combination of three elements: scaffolds, cells and morphogenic signals.

Scaffolds provide a physicochemical and biological three-dimensional microenvironment for cell growth and differentiation, promoting cell adhesion and migration [3]. Recently it was shown that biodegradable polymers (PGA/PLLA) in the form of scaffolds could provide an adequate environment for successful bioengineering of tooth crowns, which closely resemble the morphological features of natural teeth [4].

An odontogenic progenitor population was found in permanent (DPSC – Dental Pulp Stem Cells) [5] and deciduous dental pulp tissue (SHED – Stem Cells from Human Exfoliated Deciduous) [6]. These progenitor cells share two basic properties of stem cells: self-renewal and multi-lineage differentiation. DPSC have demonstrated the ability to regenerate the dentin-pulp-like complex when *in vivo* transplanted in combination with specific induction factors [5]. Comparatively, SHED have showed a significantly higher proliferation rate than bone marrow stromal stem cells (BMSSC) and DPSC, but it has failed to reconstitute a dentin-pulp-like complex [6].

Dentin matrix contains many bioactive molecules capable of stimulating tissue responses. Demineralization of dental tissues can lead to the release of growth factors following the application of cavity etching agents, restorative materials, and the carious lesion [7]. Once released, these growth factors may play key roles in signaling many of the events, including the stimulation of dental pulp stem cell differentiation [8]. EDTA treatment is considered an efficient method in dentin demineralization and it has contributed with the releasing of growth factors from the dentin matrix [9, 10]. Sodium hypochlorite is known to cause inactivation of the proteins and growth factors found in the dentin matrix [11].The removal of protein content from dentin could avoid the progenitor cell differentiation towards odontoblast phenotype, but such hypothesis was not yet tested. This study was designed to test the theory using the Tooth-Slice Scaffold Model pre-treated with NaOCl or EDTA, could interfere with differentiation process of SHED cells into odontoblast-like cells. The hypothesis was that soluble factors released by dentin EDTA treatment may play an important role in dental pulp stem cell differentiation and dentin deprotenization could prevent this differentiation.

Materials and Methods

- Sample collection - Extracted non-carious human third molars were collected from young patients (17 to 23 years old) in the department of Oral Surgery, University of Michigan, School of Dentistry. Soft tissues were removed with a periodontal scalpel and the teeth were cleaned with 70% ethanol and kept in sterile phosphate buffered saline at 4^{o} C (1X PBS)(Gibco, Invitrogen, Grand Island, NY, USA). During the experiments, cells from 4-6^{th} passage were used.

- Cell culture - Stem Cells from Human Exfoliated Deciduous Teeth (SHED - Miura *et al*., 2003) were provided by Dr. Songtao Shi, from the Dental Biology Unit, Craniofacial Skeletal Diseases Branch (NIH Bethesda, MD). The SHED were cultivated in T-75 and T-150 flasks with Dulbecco's Modified Eagle Medium low glucose (DMEM)(Gibco, Invitrogen, Grand Island, NY, USA) supplemented by 10% fetal bovine serum ES cell qualified (FBS)(Gibco, Invitrogen, Grand Island, NY, USA) and 1% penicillin-streptomycin solution (Gibco, Invitrogen, Grand Island, NY, USA), incubated at 37^{o} C in 5% CO_2.

- Preparation of tooth-slices scaffolds (TSS) - Transversally cervical slices (1 mm-thickness) were cut *per* tooth by means of a diamond saw (MK-303 Professional; MK Diamond Products, Calais, ME, USA) with coolant (1x PBS). Pulp tissue was carefully removed from the tooth-slices to prevent pre-dentin layer damage. Poly-L-latic acid solution (Boehringer Ingelheim, Germany) was prepared using 1g of PLLA particles dissolved into 20ml of chloroform (5% w/v). The tooth-slices pulp cavities were filled with 250 - 450 µm sieved sodium chloride (NaCl) and PLLA solution previously dissolved with chloroform. After polymerization, the salt was removed by distilled water, changed 4 times during 1 day.

- Experimental and control groups - Previous to PLLA scaffold preparation, 20 dentin disks were immersed in a 5.25% sodium hypochlorite solution (NaOCl) for 5 days at 4^{o}C (changed daily) in order to remove the dentin organic content. Forty tooth-slices (without NaOCl dentin pre-treatment) were placed in 1X PBS for 5 days at 4^{o}C. Just prior to cell seeding, 20 tooth-slice scaffolds were treated with 10% ethylenediaminetetraacetic acid solution (EDTA), pH 7.2, at 4^{o} C for 1 minute and washed with 1x PBS for another 1 minute. The remaining 20 tooth-slices scaffolds received no treatment (WO-T). PLLA sponges scaffolds (PSS) built without tooth-slice were used as controls (20).

- Cell seeding - The day before the cell seeding, all the TSS as well as the PSS were

disinfected and hydrophilized with sequential concentrations of ethanol (100%, 90%, 80% and 70%), for 20 minutes each, and then washed with sterile 1x PBS for 1h and kept in this buffer overnight at 4° C. When the cells reached the subconfluent stage (80%) they were removed with 0.25% trypsin-EDTA (Gibco, Invitrogen, Grand Island, NY, USA) and a total of 5 x 10^4 (20µl cell suspension) were seeded inside each PSS or TSS, placed in a 24-well plate. Samples were kept for 45 min into the incubator (37° C, 5% CO_2) for initial cell attachment to scaffolds. Then, 500µL of DMEM culture medium was added in each well plate and the scaffolds were then incubated at 37° C (Figure 1A). The culture medium was changed every other day until the RNA was extracted, according to experimental time points (7, 14, 21 and 28 days).

- *RNA extraction and RT-PCR* - The culture medium was aspirated and the samples were washed with 1X PBS. Trizol® was placed into each well plate to allow collection of the SHED RNA after 5 minutes. Scaffolds were collected in 1.0 ml Trizol® that was vortexed thoroughly. Chloroform (200 µl) was added and the samples vortexed again. The samples were centrifuged for 15 minutes (13.000 RPM at 4° C). RNA was precipitated with isopropanol (500µl), washed with 75% ethanol (500µl) and dissolved in nuclease free water (20µl). The quantity and quality (λ260/λ250) of RNA was measured by a spectrophotometer (DU 640, Beckman Coulter – CA, USA). Total RNA (0.2µg) was used in a reverse transcriptase polymerase chain reaction (SuperScript™ III Platinum®, Invitrogen) with a 54 µL reaction system including 24 µL DDW, 25 µL 2X Reaction Mix, 1 µL Taq Polymerase, 1 µL sense and 1 µL anti-sense and 2 µL template. The human-specific sense and anti-sense primers were designed according to published cDNA sequences of GenBank, and GAPDH (*sense 5' gacccctcattgacctcaact 3', antisense 5' caccaccttcttgatgtcatc 3'; 683 bp*) was used as house-keeping gene to normalize RNA expression. SHED differentiation was monitored for three odontoblast markers: (DSPP) dentin sialophosphoprotein (*sense 5' gacccctcattgacctcaact 3', antisense 5' tgccatttgctgtgatgttt 3'; 181 bp*), (DMP-1) dentin matrix protein-one (*sense 5'caggagcacaggaaaaggag 3', antisense 5' ctggtggtatcttgggcact 3'; 213 bp*) and (MEPE) matrix extracellular phosphoglycoprotein (sense 5' gcaaaagcacccatcgtatt 3', antisense 5' ctgccctctacaaggctgac 3'; 385 bp). RNA collected from human odontoblasts (fresh extracted third molars) was used as positive control for DSPP, MEPE and DMP-1 expression. The following Polymerase Chain Reaction (PCR) cycles was used: denaturation, 94° C for 45s; annealing, 57° C for 45s; and extension, 72° C for 60s, for

35 cycles, then 72° C for 5 min and held at 4° C. The PCR products were separated by using 1.5% agarose gel electrophoresis and were stained with ethidium bromide; digital images were taken using an ultraviolet background. Three independent experiments were performed to verify the reproducibility of the results.

- *WST-1 proliferation assay* - TSS and PSS were prepared as previously described. SHED (5 x 10^4) were seeded into the TSS and PSS and cultured in 24-well plates (37° C in 5% CO_2). WST-1 cell proliferation reagent was added to the cells in a 1:10 final dilution. After one-hour incubation (37° C in 5% CO_2), 100μL of culture medium/WST-1 reagent were transferred to a 96-well plate in order to read the level of absorbance in a ELISA multiplate reader (TECAN, Genius USA-IL) with a wavelength of 450nm. The measurements were performed after 7, 14, 21 and 28 days of cell cultured. Data were submitted to statistical analysis using one-way ANOVA followed by Tukey's test (Sigmastat 2.0 software, SPSS, Chicago, IL, USA), at $p<0.05$.

- *Implantation* – SHED (5 x 10^5) were prepared in a cell suspension solution (20 μl) using Matrigel (BD Biosciences, Bedford, MA, USA) and DMEM (1:1), and seeded inside the TSS (NaOCl, EDTA and WO-T) and PSS. The scaffolds (n=2 each group, 2 for animal) were transplanted subcutaneously into the dorsum of 5-7 week-old male SCID mice (n=8) (CB.17 SCID; Charles River, Wilmington, MA, USA) (Figure 2A). The study was approved by the animal care ethics committee of the University of Michigan (Ann Arbor, MI – USA), using UCCA and IRB approved protocols. Two tooth slice scaffolds were implanted per mice. After 14 and 28 days, the mice were euthanized. The implants were retrieved (Figure 2D), the RNA was extracted and RT-PCR was performed as previous described.

Results

Analysis of temporal gene expression by RT-PCR in SHED cultured in TSS model

RT-PCR demonstrated that SHED altered their genetic expression during culture periods (Figure 1C). Transcripts for DSPP, DMP-1 and MEPE were detected by RT-PCR using total RNA isolated from the scaffolds after the time course. DSPP, DMP-1 and MEPE mRNA began their expression in cells cultured in TSS treated with EDTA or WO-T after 14 days, being also expressed at the 21 and 28-day intervals. No expression was found by the 7th day culture for all groups. No mRNA expression for differentiation markers was found in TSS treated with NaOCl or in PSS, for any time point.

Effect of dentin pre-treatment on SHED proliferation

The statistical analysis showed interactions between the time course and experimental groups. For all time points (7, 14, 21 and 28 days), SHED cultivated in PSS showed higher rate of proliferation compared to NaOCl, WO-T and EDTA groups (p<0.05). After 14 days, SHED cultivated in TSS NaOCl treated presented bigger proliferation rate than cells cultured in TSS WO-T and TSS EDTA treated, and this pattern was maintained thereafter (p<0.05). SHED cultured in TSS WO-T and EDTA treated groups, reached a peak value on day 14, and then decreased (Table 1; Figure 1B).

DSPP, DMP-1 and MEPE mRNA expression after in vivo SHED transplantation

SHED seeded in the TSS or PSS were implanted into SCID mice. Transcript for DMP-1 was found in TSS EDTA treated or TSS WO-T group after 14 days. MEPE positive cells were only found in EDTA treated group. In 28 days implantation, DSPP, DMP-1 and MEPE were expressed by SHED cultured in both TSS EDTA treated and TSS WO-T. No reactivity of differentiation markers was found in TSS NaOCl treated or in PSS, in any evaluated time point (Figure 2E).

Discussion

After tooth formation, the dental pulp maintains its defensive and regenerative ability. Evidence shows that human dental pulp has a high proliferative and multi-lineage subpopulation of cells capable of differentiating in many directions, according to stimulation [5, 6].

The dentine matrix contains growth factors and cytokines that are sequestered during dentinogenesis [12]. Following physiological stimulation or injury, such as caries, trauma and operative procedures, these molecules are released by acids with others extracellular matrix components and induce reparative dentinogenesis [13].

The tissue engineering is based on a principle whereby undifferentiated cells placed into a biocompatible matrix respond to specific signals which causes proliferation and differentiation. Here we use SHED cultured in biodegradable polymer scaffolds fabricated inside the dental pulp cavity of a 1 mm thick tooth-slice to mimic the dentin environment that provides bioactive molecules for cell differentiation. The tooth-slice model has been used to study human dental pulp angiogenesis and is a useful tool for research therapeutic strategies for avulsed immature teeth [14].

In the present study we utilized tooth-slice scaffolds to study SHED differentiation. According to Roberts-Clark and Smith (2000) [9], EDTA has the ability to extract bioactive and extracellular molecules (ECM) from dentin. We hypothesized that bioactive

molecules sequestered in dentin matrix could be released by EDTA action and promote dental pulp stem cells differentiation.

The results from our *in vitro* experiment confirm that SHED cultured in tooth-slice scaffolds altered their genetic expression during the time course. Tooth-slice scaffolds, which were EDTA treated or remain without treatment, expressed differentiation markers (DSPP, DMP-1 and MEPE) after 14 days and maintained expression thereafter. Interestingly, The expression of differentiation markers for untreated group followed the pattern of EDTA group. We speculate that the PLLA based scaffold used in the present study releases lactic acid as a product of degradation over the time, and may act as dentin conditioning agent, which promotes growth factors release.

To eliminate the effect of dentin matrix molecules on dental pulp stem cells we used NaOCl to remove the organic content from the dentin. Sodium hypochlorite (5.25%) is capable of dissolving the entire organic component of dentin [11]. Additionally, SHED seeded in PLLA sponge scaffolds with no tooth-slice were used as negative control for the expression of differentiation markers. No reactivity of differentiation markers was found in tooth-slices treated with NaOCl or in PSS during time course evaluation. For the PSS, there was a lack of inductive signals without the dentin disks. Similarly, when the dentin had the protein components removed by NaOCl treatment, there was no inductive signal present, probably because the growth factors sequestered into the dentin matrix were denature with the NaOCl treatment.

The first evidence that human pulp cells of odontoblasts-lineage can differentiate in odontoblasts *in vitro* upon contact with dentin chemically treated utilized scanning electronic microscopy (SEM) images and morphologic analysis to draw conclusions regarding the differentiation status of these cells [15]. However, using only morphological analysis is not a reliable method to confirm cell differentiation itself. Usually, isolated stem cells from human dental pulp (from permanent and deciduous teeth) have the typical fibroblastic morphology, spindle-shaped with extending cytoplasmatic processes. The image of a process extending into the opening of a dentinal tubule can occur only by chance.

In the present study, RT-PCR with three markers for odontoblast differentiation were used: dentin sialo-phosphoprotein (DSPP), dentin matrix protein 1 (DMP-1) and matrix extracellular phosphoglycoprotein (MEPE).

DSP and DPP are cleaved products of DSPP, which is a highly phosphorylated noncollagenous protein secreted by odontoblasts and is a dentin-specific marker [16].

However, DSPP is also expressed in bone [17]. Western blots and RT-PCR indicated that DSPP gene is expressed at a lower level in bone than in dentin (1/400). Therefore, DSPP may act as a phenotypic marker of odontoblast-like differentiation.

DSPP and DMP-1 are expressed by differentiating odontoblasts [18, 19, 20]. 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 [18].

MEPE is a member of bone matrix protein family and involved in regulation of bone metabolism [21, 22]. Bone marrow mesenchymal stem cells cultured in osteoinduction medium increased MEPE mRNA expression in a time-dependent manner [21, 22]. However, the expression of mRNA MEPE in dental pulp stem cells has been showed contradictory data. According to Liu *et al*. (2005) [23] MEPE is downregulated as dental pulp stem cells differentiated, while DSP was upregulated. Otherwise, quantitative RT-PCR revealed that DSPP and MEPE expression increased time dependently in induction cultures, showing a similar regulatory pattern in dental pulp stem cells differentiation [24].

In our *in vitro* experiment, DSPP, DMP-1 and MEPE markers appeared after 14 days, following the SHED differentiation. However, the *in vivo* experiment revealed differences in temporal gene expression. Differentiation markers were detected after 14 days of tooth-slice scaffold implantation. DMP-1 marker was found in cells when the tooth-slice scaffolds were treated with EDTA or even when no dentin treatment was performed. MEPE positive cells were only found in tooth-slice scaffolds treated with EDTA, and no DSPP expression was found in 14 days transplantation. The transcripts for the three markers were detected after a 28-day period. In a previous study, DPSCs were transplated in conjunction with HA/TC powder into immunocompromiced mice. After 6 weeks post transplantation, DSPCs generated a dentin-like-structures lining the HA/TC surfaces. Furthermore, the DPSC transplants expressed transcripts for DSPP.

Comparing the results of the both time course assays (*in vitro* and *in vivo*) revealed a “delay” of differentiation markers expression when the RNA was collected from the implants. The differences observed might be related with lack of nutrients that the transplanted cells were exposed to at first. Even using Matrigel, a soluble extract of basement membrane loaded with proteins that serve as a source of nutrients for cell survival, and seeding a higher number of cells ($5x10^5$), a significant proportion of these cells usually die during the transplantation procedures.

The results of cell proliferation assay showed that the number of cells decreased following the temporal expression of odontoblast differentiation markers. SHED cultured

in tooth-slice scaffolds treated with EDTA or untreated reached a peak value on day 14, and then decreased, corroborating with the results of time course *in vitro* differentiation. The other groups, tooth-slice scaffolds NaOCl treated and PLLA sponge scaffold, continued their proliferation until 21 and 28 days, respectively.

Under clinical perspectives, the transition from deciduous to permanent teeth is an exclusive physiologic event in which the formation and eruption of permanent teeth is coordinate with resorption of the roots of primary teeth [6]. This process occurs in 6 to 12 year old children. At this age, the prevalence of dental trauma is high and frequently leads to an irreversible pulp inflammation or necrosis [25]. Pulp necrosis in young permanent teeth stops the dentin formation and results in incomplete vertical and lateral root development [26]. The current endodontic available treatment (*i.e.* calcium hydroxide followed by canal fulfillment with gutta-percha) eliminates the bacterial infection, but does not allow for completion of root formation, resulting in a fragile root tooth structure with thin lateral walls susceptible to fracture by secondary trauma.

The future goal for regenerative endodontics, under a biological perspective, will be using the cells from own patient (available sources) associated with biodegradable materials (scaffolds) created inside the endodontic canal of a young traumatized permanent tooth, in order to recovery the vitality and the function. Here we showed that scaffolds built inside the toot-slice cavity and seeded with SHED provide an adequate environment for cell growth and differentiation, increasing the perspectives to use this model to study dental pulp regeneration.

Conclusions

- Tooth-slice scaffold model provides an adequate environment for SHED growth and differentiation;
- RT-PCR demonstrated that SHED cultured in tooth-slice scaffolds (*in vitro* and *in vivo*) altered their genetic expression during the time course;
- Cell proliferation rate was reduced following the SHED differentiation;
- The overall findings demonstrated that untreated or EDTA treated dentin allowed for the differentiation processes, while the pre-treatment with sodium hypochlorite exhibited an inhibitory effect, preventing the SHED differentiation.

References

1. Nör JE. Tooth Regeneration in Operative Dentistry. Oper Dent. 2006 Nov-Dec;31(6):633-42.

2. Langer R, Vacanti JP. Tissue engineering. Science. 1993 May 14;260(5110):920-6.

3. Nakashima M, Akamine A. The application of tissue engineering to regeneration of pulp and dentin in endodontics. J Endod. 2005 Oct;31(10):711-8.

4. Young CS, Terada S, Vacanti JP, Honda M, Bartlett JD, Yelick PC. Tissue engineering of complex tooth structures on biodegradable polymer scaffolds. J Dent Res. 2002 Oct;81(10):695-700.

5. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000 Dec 5;97(25):13625-30.

6. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A. 2003 May 13;100(10):5807-12.

7. Murray PE, Smith AJ. Saving pulps--a biological basis. An overview. Prim Dent Care. 2002 Jan;9(1):21-6.

8. Tziafas D. The future role of a molecular approach to pulp-dentinal regeneration. Caries Res. 2004 May-Jun;38(3):314-20.

9. Roberts-Clark DJ, Smith AJ. Angiogenic growth factors in human dentine matrix. Arch Oral Biol. 2000 Nov;45(11):1013-6.

10. Graham L, Cooper PR, Cassidy N, Nor JE, Sloan AJ, Smith AJ. The effect of calcium hydroxide on solubilisation of bio-active dentine matrix components. Biomaterials. 2006 May;27(14):2865-73.

11. Beltz RE, Torabinejad M, Pouresmail M. Quantitative analysis of the solubilizing action of MTAD, sodium hypochlorite, and EDTA on bovine pulp and dentin. J Endod. 2003 May; 29(5):334-7.

12. Smith AJ, Lesot H. Induction and regulation of crown dentinogenesis: embryonic events as a template for dental tissue repair? Crit Rev Oral Biol Med. 2001;12(5):425-37.

13. Tziafas D, Smith AJ, Lesot H. Designing new treatment strategies in vital pulp therapy. J Dent. 2000 Feb;28(2):77-92.

14. Gonçalves SB, Dong Z, Bramante CM, Holland GR, Smith AJ, Nör JE. Tooth slice-based models for the study of human dental pulp angiogenesis. J Endod. 2007 Jul;33(7):811-4.

15. Huang GT, Shagramanova K, Chan SW. Formation of odontoblast-like cells from cultured human dental pulp cells on dentin in vitro. J Endod. 2006 Nov;32(11):1066-73.

16. MacDougall M, Simmons D, Luan X, Nydegger J, Feng J, Gu TT. Dentin phosphoprotein and dentin sialoprotein are cleavage products expressed from a single transcript coded by a gene on human chromosome 4. Dentin phosphoprotein DNA sequence determination. J Biol Chem. 1997 Jan 10;272(2):835-42.

17. Qin C, Brunn JC, Cadena E, Ridall A, Tsujigiwa H, Nagatsuka H, Nagai N, Butler WT. The expression of dentin sialophosphoprotein gene in bone. J Dent Res. 2002 Jun;81(6):392-4.

18. D'Souza RN, Cavender A, Sunavala G, Alvarez J, Ohshima T, Kulkarni AB, MacDougall M. Gene expression patterns of murine dentin matrix protein 1 (Dmp1) and dentin sialophosphoprotein (DSPP) suggest distinct developmental functions in vivo. J Bone Miner Res. 1997 Dec;12(12):2040-9.

19. Bègue-Kirn C, Krebsbach PH, Bartlett JD, Butler WT. Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. Eur J Oral Sci. 1998 Oct;106(5):963-70.

20. Bleicher F, Couble ML, Farges JC, Couble P, Magloire H. Sequential expression of matrix protein genes in developing rat teeth. Matrix Biol. 1999 Apr;18(2):133-43.

21. Petersen DN, Tkalcevic GT, Mansolf AL, Rivera-Gonzalez R, Brown TA. Identification of osteoblast/osteocyte factor 45 (OF45), a bone-specific cDNA encoding an RGD-containing protein that is highly expressed in osteoblasts and osteocytes. J Biol Chem. 2000 Nov 17;275(46):36172-80.

22. Zhang GX, Mizuno M, Tsuji K, Tamura M. Regulation of mRNA expression of matrix extracellular phosphoglycoprotein (MEPE)/ osteoblast/osteocyte factor 45 (OF45) by fibroblast growth factor 2 in cultures of rat bone marrow-derived osteoblastic cells. Endocrine. 2004 Jun;24(1):15-24.

23. Liu H, Li W, Shi S, Habelitz S, Gao C, Denbesten P. MEPE is downregulated as dental pulp stem cells differentiate. Arch Oral Biol. 2005 Nov;50(11):923-8.

24. Wei X, Ling J, Wu L, Liu L, Xiao Y. Expression of mineralization markers in dental pulp cells. J Endod. 2007 Jun;33(6):703-8.

25. Roberts G & Longhurst P. Oral and dental trauma in children and adolescents. 1st Edition. Oxford University Press, 1996: 1-9.

26. Andreasen JO, Borum MK, Jacobsen HL, Andreasen FM. Replantation of 400 avulsed permanent incisors. 2. Factors related to pulpal healing. Endod Dent Traumatol. 1995 Apr;11(2):59-68.

Table 1. Proliferation of SHED cultured in the tooth-slice scaffold model. Means of optical density measured in a time course assay.

Time (days)	Group							
	PSS		NaOCl		WO-T		EDTA	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd
7	0,980 ^{Ca}	0,023	0,617 ^{Bb}	0,016	0,594 ^{Ab}	0,021	0,590 ^{Ab}	0,013
14	1,402 ^{Ba}	0,018	0,911 ^{Ab}	0,050	0,720 ^{Ac}	0,047	0,682 ^{Ac}	0,029
21	1,778 ^{Aa}	0,082	1,012 ^{Ab}	0,081	0,684 ^{Ac}	0,067	0,548 ^{Ac}	0,059
28	1,882 ^{Aa}	0,090	0,988 ^{Ab}	0,082	0,514 ^{Ac}	0,058	0,406 ^{Bc}	0,017

One-way ANOVA followed by Tukey's test. Means followed by uppercase in the columns and lowercase in the lines show the statistical differences.

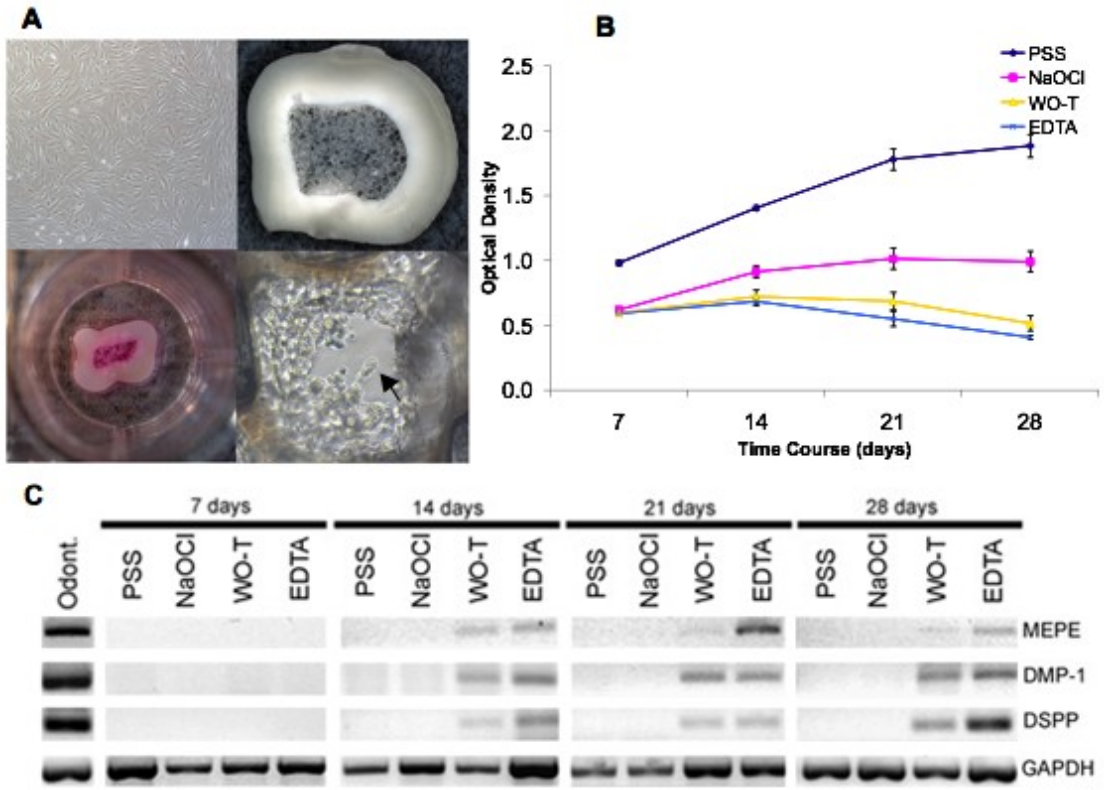

Figure 1. (A) Tooth-slice scaffold model to study the SHED differentiation. PLLA scaffolds were built inside the pulp chamber of tooth-slices. Cells ($5x10^4$) were seeded in the tooth-slice scaffold with different dentin pre-treatment (NaOCl or EDTA), no treatment (WO-T), or in a PLLA sponge scaffold without tooth-slice (PSS), and cultured in 24-well plate (arrow indicates cells growing inside the scaffold porous). **(B)** To evaluate the SHED proliferation, the WST-1 proliferation assay was preformed after 7, 14, 21 and 28 days. SHED cultivated in PSS showed higher rate of proliferation compared to NaOCl, WO-T and EDTA groups ($p<0.05$). After 14 days, SHED cultivated in TSS NaOCl treated presented bigger proliferation rate than cells cultured in TSS WO-T and TSS EDTA treated ($p<0.05$). **(C)** To analyze the expression of odontoblast differentiation markers by SHED cultured in tooth-slice scaffolds under different dentin pre-treatments, RNA was collected by Tryzol® and RT-PCR was performed after different periods (7, 14, 21 and 28 days). DSPP, DMP-1 and MEPE positive cells were found in tooth-slice scaffolds treated with EDTA or WO-T after 14 days and maintained the expression up to 28 days. No reactivity of differentiation markers was found in tooth-slices treated with NaOCl or in PLLA sponge scaffolds.

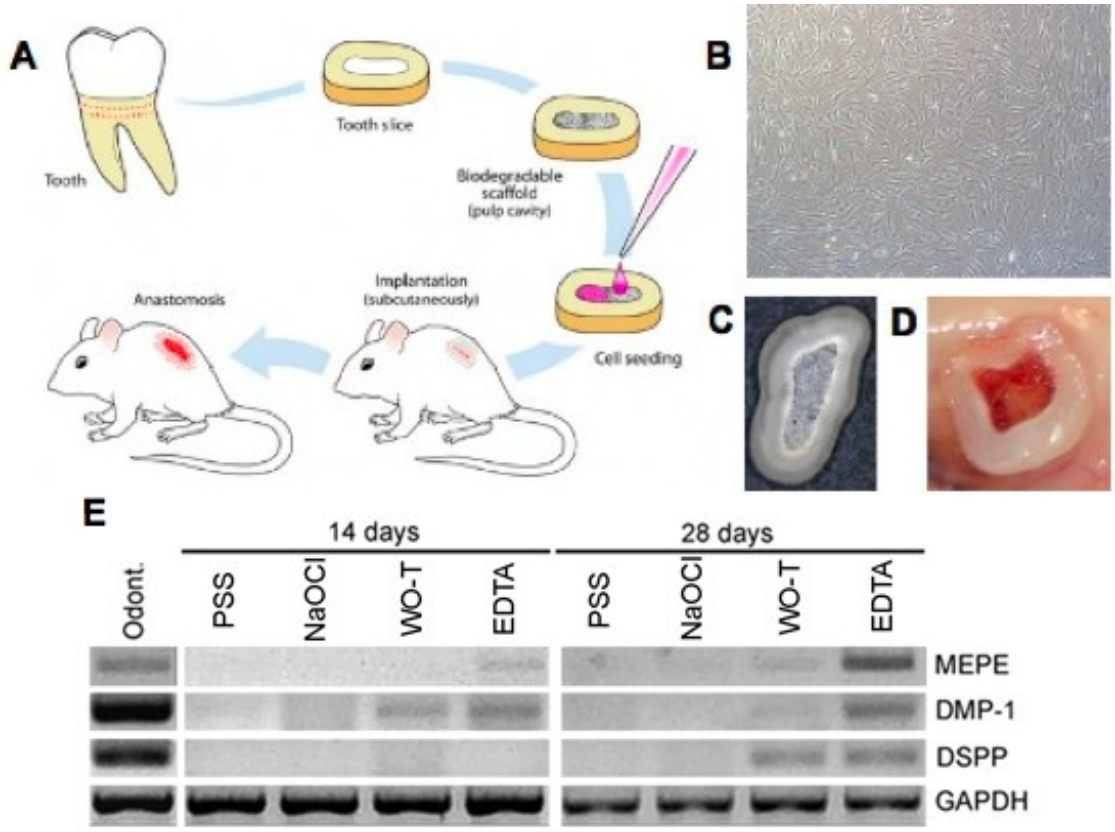

Figure 2. (A) Tooth-slice scaffold model to study i*n vivo* SHED differentiation (schematic diagram). **(B and C)** SHED ($5x10^4$) were seeded in PLLA biodegradable scaffold prepared inside the pulp chamber of dentin disks. The tooth-slice scaffolds containing cells were transplanted into SCID mice. **(D)** After 14 and 28 days, the implants were retrieved and RNA was collected by Tryzol® and RT-PCR was performed using DSPP, DMP-1 and MEPE human primers. **(E)** Transcripts for DMP-1 were found in TSS treated with EDTA or WO-T after 14 days. MEPE positive cells were only found in TSS treated with EDTA. After 28 days, differentiation markers (DSPP, DMP-1 and MEPE) were found both in EDTA and WO-T groups. No expression of the odontoblast differentiation markers was found when the cells were seeded in the tooth-slice NaOCl treated or in PLLA sponge scaffold groups.

4. ARTIGO 2

Effect of dentin-derived BMPs on SHED differentiation

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Key words: tissue engineering; stem cell from human deciduous teeth (SHED); scaffolds; dentin; bone morphogenetic protein (BMP); differentiation.

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Abstract

Tissue engineering is based on the triad cells, signals and scaffolds. Here we used stem cells from human exfoliated deciduous teeth (SHED) seeded into tooth-slice scaffolds (TSS) to study the role of dentin-derived BMPs on cells differentiation. Dentin disks (1 mm thickness) were prepared from freshly extracted non-carious human third molars. Biodegradable highly porous poly-L-latic acid (PLLA) scaffolds were fabricated inside the pulp cavity of the tooth-slices. To verify the dentin-derived BMPs bioactivity, SHED ($5x10^4$) were cultured in TSS in the presence of 2 μg/ml of anti-human BMP-2, -4, and -7 antibodies for 14 days. Cells cultured in PLLA sponges scaffolds with no tooth-slice (PSS) and in TSS EDTA treated (without antibodies) were used as controls. Additionally, SHED ($5 X10^5$) cultured in 24-well plate were treated with 100ng/mL of rhBMP-2; -4; or -7 for 24 hours. RNA was collected with Tryzol® and RT-PCR was performed to evaluate the expression of odontoblast differentiation markers (DSPP, DMP1 and MEPE). BMP-2/4Ab prevented the expression of differentiation markers and no inhibitory effect was detected for BMP-7Ab. After 24 hours, expression of DSPP, DMP-1 and MEPE was found in SHED treated by rhBMP-2, and DSPP and DMP-1 for rhBMP-4 and rhBMP-7, but not for untreated cells. The results suggest that the dentin-derived morphogenic signals BMP-2 and -4 play an important role in dental pulp stem cell differentiation in a TSS model.

Introduction

Dentinal repair occurs through the activity of specialized cells, called odontoblasts, which are thought to be maintained by as undefined precursor population associated with pulp tissue [1]. Studies have shown that the dental pulp from both permanent and deciduous teeth contains a progenitor cell population capable of self-renewal and multi-lineage differentiation [2, 3, 4].

Moderate carious lesions stimulate the secretory activity of the odontoblasts resulting in reactionary dentine [1, 5], while deep cavity preparation or severe carious lesions may lead to partial destruction of the odontoblastic layer. These conditions attract pulp cells to the injury site promoting their differentiation into odontoblast-like cells to replace the necrotic odontoblasts and secrete a reparative dentine matrix [6].

Although the mechanisms underlying the reparative dentin formation have not been complete elucidated, many growth factors and extracellular matrix proteins, such as bone morphogenetic proteins (BMPs), play an important role to induct progenitors cells differentiation after pulp injury [7, 8, 9].

Bone morphogenetic proteins are members of the transforming growth factor β (TGF-β) superfamily, that were originally identified as protein regulators of cartilage and bone formation and have been involved in embryogenesis and morphogenesis of various organs and tissues, including teeth [10]. Human recombinant bone morphogenetic proteins (rhBMP-2, rhBMP-7) induce dentinogenesis [7, 8, 9, 11]. Recently, it was discovered that dentin extracts induce differentiation of dental pulp stem cells [12]. However, the role of the dentin-derived (not recombinant proteins) BMP-2, BMP-4 or BMP-7 on the differentiation of dental pulp stem cells is not fully understood. The purpose of this study was to analyze the effect of dentin-derived bone morphogenetic proteins on SHED differentiation.

Materials and Methods

- *Cell culture* - Stem Cells from Human Exfoliated Deciduous Teeth (SHED) and Dental Pulp Stem Cells from permanent teeth (DPSC) were provided by Dr. Songtao Shi from the Dental Biology Unit, Craniofacial Skeletal Diseases Branch (NIH Bethesda, MD). SHED and DPSC were cultivated in Dulbecco's Modified Eagle Medium (DMEM) low glucose (Gibco, Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) ES cell qualified (Gibco, Invitrogen, Grand Island, NY, USA) and 1% penicillin-

streptomycin solution (Gibco, Invitrogen, Grand Island, NY, USA) incubated at 37° C in 5% CO_2. Fibroblast cells, derived from dental pulp, were retrieved by overgrowth method. Fibroblasts, MG-63 and UMSCC 11A were cultured with DMEM high glucose (Gibco, Invitrogen, Grand Island, NY, USA) supplemented by 10% FBS and 1% antibiotics. Human Dermal Microvessel Endothelial Cells (HDMEC) were cultured in EGM2-MV (Cambrex, Walkersville, MD, USA).

- Protein extraction and western blot - Cells were removed with 0.25% trypsin-EDTA, lyzed in a buffer system (NP-40 protein lysis buffer) sonicated, centrifuged and the supernatant was collected. This protein was quantified in an ELISA multiplate reader (TECAN, Genius) using a wavelength of 585nm (Magellan program).

Each sample (20μL) was added to a Mix buffer (4X Load buffer and 0.5M DDT) and incubated at 90-95° C for 5 minutes. The samples were separated on a 10% NuPAGE gel (Invitrogen), transferred onto polyvinylidene fluoride membranes and blocked with 5% (w/v) non-fat dry milk in TBST for 1 hour at room temperature. The membranes were then incubated at 4°C overnight with the following antibodies against specific human proteins: Monoclonal Anti-human BMPR-IA antibody (MAB2406, R&D Systems, Inc); Monoclonal Anti-human/mouse BMPR-IB/ALK-6 antibody (MAB505, R&D Systems, Inc); and Monoclonal Anti-human BMPR-II antibody (MAB811, R&D Systems, Inc). All antibodies were reconstituted with sterile phosphate buffered saline (1X PBS) and used in a 1:500 dilution. The membranes were then washed (TBST) and incubated with anti-mouse IgG for 2hs at room temperature, followed by being washed and reacted with color development Super Signal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc Rockford, IL) according to the manufacturer's recommendations. Signals were visualized with Kodak BIOMAX MR films (Kodak, Rochester, NY, USA).

- Sample collection - Freshly extracted non-carious human third molars were collected from 17 to 23 years old patients in the Department of Oral Surgery (School of Dentistry, University of Michigan, USA). Soft tissues were removed with a periodontal scalpel and the teeth were disinfected with 70% ethanol and kept in 1X PBS at 4° C.

- Preparation of tooth-slices scaffolds (TSS) - Third molars were sectioned transversally at the cement-dentin junction (1 mm thickness) with a diamond saw (MK-303 Professional; MK Diamond Products, Calais, ME, USA) with coolant (1X PBS). Pulp tissue was carefully removed from the slices to prevent pre-dentin layer damage. Poly-L-latic acid solution (Boehringer Ingelheim, Germany) was prepared using 1g of PLLA particles dissolved into 20 ml of chloroform (5% w/v). The pulp cavities were filled up

with 250 - 450 µm sieved sodium chloride (NaCl) and soaked up the PLLA solution, previously dissolved with chloroform. After polymerization, the salt was removed by distilled water, changed 4 times during 1 day.

- *Experimental and control groups* - The specific effect of dentin-derived BMP-2, -4 and -7 on SHED differentiation was evaluated using 2 µg/ml of anti-human BMP-2/4 antibody (MAB3551, R&D Systems), or 2 µg/ml anti-human BMP-7 antibody (MAB3541, R&D Systems), which was added to the culture medium to neutralize the dentin-derived BMP's bioactivity. The culture medium and the neutralized antibodies were changed every other day during 14 days. Three experimental groups were tested: G1 – SHED-TSS culture in presence of BMP-2/4Ab; G2 – SHED-TSS culture in presence of BMP-7Ab; and G3 – SHED-TSS culture in presence of BMP-2/4Ab and BMP-7Ab associated. SHED cultured in PLLA sponge scaffolds without dentin-slices (PSS) and TSS treated with EDTA (without antibody) were used as negative and positive control, respectively.

- *Cell seeding* - The day before the cell seeding, all the TSS and PSS were disinfected and hydrofilized with sequential concentrations of ethanol (100%, 90%, 80% and 70%), immersed for 20 minutes in each concentration. They were washed with sterile 1X PBS for 1 hour and stored at 4° C overnight. SHED (5 x 10^4 cells in 20µl suspension) were seeded into each PSS or TSS (Figure 1). Samples were kept for 45 min into the incubator (37° C in 5% CO_2) for initial cell attachment to scaffolds. Culture medium (500µL) was added to each well plate and the samples incubated. The culture medium was changed every other day until RNA extraction was performed.

- *Recombinant human bone morphogenetic protein (rhBMP-2, -4 and -7)* - SHED (5 X10^5) were cultured in a 24-well plate (37° C in 5% CO_2) and after they reached 80% confluency they were treated with 100ng/mL of recombinant bone morphogenic proteins (G1: rhBMP-2; G:2 rhBMP-4; G:3 rhBMP-7 and Control: no treatment). After 24hs, the RNA was collected and RT-PCR performed.

- *RNA extraction and RT-PCR* - The culture medium was aspirated and the samples were washed with 1X PBS. Trizol® was placed into each well plate for 5 minutes. Scaffolds were collected in 1.0ml Trizol®, vortexed thoroughly; chloroform (200µl) was added and the samples were vortexed again. The samples were centrifuged for 15 minutes (13.000 RPM at 4°C). RNA was precipitated with isopropanol (500µl), washed with 75% ethanol (500µl) and dissolved in nuclease free water (20µl). The quantity and quality (λ260/λ280) of RNA was measured by a spectrophotometer (DU 640, Beckman Coulter – CA, USA). Total RNA (0.2µg) was used in a RT-PCR (SuperScript™ III

Platinum®, Invitrogen) with a 54 μL reaction system including 24 μL DDW, 25 μL 2X Reaction Mix, 1 μL Taq Polymerase, 1 μL sense and 1 μL anti-sense and 2 μL template. The human-specific sense and anti-sense primers were designed according to published cDNA sequences of GenBank, and GAPDH (*sense 5' gacccttcattgacctcaact 3', antisense 5' caccaccttcttgatgtcatc 3'; 683 bp*) was used as house-keeping gene to normalize RNA expression. SHED differentiation was monitored for three odontoblasts markers: (DSPP) dentin sialophosphoprotein (*sense 5' gacccttcattgacctcaact 3', antisense 5' tgccatttgctgtgatgttt 3'; 181 bp*), (DMP-1) dentin matrix protein one (*sense 5'caggagcacaggaaaaggag 3', antisense 5' ctggtggtatcttgggcact 3'; 213 bp*) and (MEPE) matrix extracellular phosphoglycoprotein (sense 5' gcaaaagcacccatcgtatt 3', antisense 5' ctgccctctacaaggctgac 3'; 385 bp). RNA collected from human odontoblasts (fresh third extracted molars) was used to control for DSPP, MEPE and DMP-1 expression. The following Polymerase Chain Reaction (PCR) cycles was used: denaturation, 94°C for 45s; annealing, 57°C for 45s; and extension, 72°C for 60s, for 35 cycles, then 72°C for 5 min and hold in 4°C. The PCR products were separated by using 1.5% agarose gel electrophoresis and were stained with ethidium bromide; digital images were taken using an ultraviolet background. Three independent experiments were performed to verify the reproducibility of results.

Results

BMPs receptors expressed by dental pulp cells – The bone morphogenetic protein (BMP) receptors are a family of transmembrane serine/threonine kinases that include the type I receptors (BMPRIA and BMPRIB – 50/55 KDa) and the type II receptor (BMPR2 – 70/80 KDa). The expression of BMPs receptors was characterized by western blot (Figure 2). HDMEC, MG-63 and UM SCC 11A were used as controls for BMPs receptors expression. The dental pulp cells (SHED, DPSC and fibroblast) characterized by western blot expressed the three BMPs receptors, BMPR-IA, BMPR-IB and BMP-II.

Effect of rhBMP-2, -4 and -7 on SHED mRNA expression – In an attempt to find correlating evidence regarding BMP induced SHED differentiation, we tested whether rhBMPs could stimulate the expression of odontoblast differentiation markers (DSPP, DMP-1 and MEPE) in stem cells from human exfoliated deciduous teeth. RT-PCR analysis revealed the expression of human DSPP and DMP-1 markers for rhBMP-2, rhBMP-4 and rhBMP-7. Transcripts for MEPE were only detected in cells treated with

rhBMP-2 (Figure 3). Human dental pulp stem cells without rhBMP treatment did not express RNA differentiation markers.

Effect of dentin-derived morphogenic BMPs on SHED differentiation – This experiment determined the availability and bioactivity of dentin inducers in the stem cell differentiation. SHED cells were seeded into tooth-slice scaffolds and cultured for 14 days in the presence of BMP neutralizing antibodies. RT-PCR analysis showed no expression of differentiation markers (DSPP/DMP-1/MEPE) when BMP-2/4Ab was added to the culture medium. When BMP-7Ab was added to the culture medium, the expression of differentiation markers (DSPP, DMP-1 and MEPE) was detected by RT-PCR showing similar pattern than that observed in the tooth-slice scaffolds treated with EDTA. Human dental pulp stem cells seeded into PSS (no addition of neutralized antibodies) did not express transcripts for human DSPP, DMP-1 or MEPE (Figure 4).

Discussion

The goal of conservative endodontics treatments is to restore or regenerate the dentin-pulp-complex in order to maintain the vitality and function of teeth. Calcium hydroxide-based agents has been used as direct pulp capping material because it can stimulate pulpal tissue to produce reparative dentin, however the quality of the newly formed dentin is questionable since dentin is often porous [13].

In an attempt to apply more biological approaches in conservative treatments of pulp tissue, new techniques and technologies have been suggested. Since the recent approval of using BMPs for bone healing in fractures by the US Food and Drug Administration, the application of bioactive molecules in endodontics and periodontal surgery assumed relevant issue of research and became a potential clinical treatment approach in the near term.

At least four distinct families of morphogens are involved in embryonic craniofacial morphogenesis, but BMPs seems to be sufficient for dental tissue regeneration [14]. The role of BMPs in dental tissues has been intensively studied. The morphogens BMP-2, BMP-4, BMP-6, BMP-7 and Gdf11 are inductive signals that act as growth/differentiation factors during odontoblast differentiation. The expression of BMP-2 is upregulated during the terminal differentiation of odontoblasts [8, 14]. BMP-7 has been shown to promote reparative dentinogenesis and pulp mineralization in animal model [15, 16]. Recombinant human bone morphogenetic proteins (rhBMP) have shown to induce reparative/regenerative dentin formation *in vivo* [8, 9, 15].

The response of dental pulp cells to BMPs suggests that the cells present receptors for these bioactive molecules. The bone morphogenetic protein receptors (BMPR) are a family of trans-membrane serine/threonine kinases that include the type I receptors (BMPR-IA and BMPR-IB - 50-55 kD), and the type II receptor (BMPR-II - 70-80 kD). In the present study, the dental pulp cells (SHED, DPSC and fibroblast) characterized by western blot expressed the BMPR-IA, BMPR-IB and BMP-II receptors.

To verify the influence of rhBMP and dentin-derived BMPs on SHED differentiation, RT-PCR was used for expression of odontoblast differentiation markers: dentin sialo-phosphoprotein (DSPP), dentin matrix protein 1 (DMP-1) and matrix extracellular phosphoglycoprotein (MEPE). DSPP is a phosphorylated parent protein that is cleaved post-transcriptionally into two proteins, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) [17, 18]. DSPP expression is well established as a marker of odontoblastic differentiation [19, 20, 21]. Indeed, DSPP was used by Songtao Shi and his research group during the initial characterization of the dental pulp stem cell [2, 4]. The same group has also used DSPP to differentiate the processes of dentinogenesis, induced by these cells, from osteogenic processes induced by bone marrow stromal stem cells [22]. Recent studies have shown that DSPP gene is expressed in bone, although DSPP expression is at a much lower level in osteoblasts than in odontoblast-like cells [1/400] [23]. DMP-1 is expressed by differentiating odontoblasts [24, 25] and plays a role in the mineralization of dentin since the expression of DMP-1, as well as DSPP, that was detected in functional odontoblasts prior to mineralization [26]. MEPE is a member of bone matrix protein family and involved in regulation of bone metabolisms [27, 28] and recently was found being expressed in the early odontoblast-like differentiation, increased time dependently in induction cultures [29].

In the present research SHED were tested whether rhBMPs could stimulate the expression of odontoblast differentiation markers. No transcripts for DSPP, DMP-1 or MEPE were detected in control group (untreated cells). In contrast, the treatment of rhBMP-2, rhBMP-4 and rhBMP-7 on SHED revealed the expression of human DSPP and DMP-1, but transcripts for MEPE were only detected in cells treated with rhBMP-2. The results suggest that the SHED may be more reactive to BMP-2, since the same amount of recombinant protein was used for all tested groups.

Studies have been shown that the dentin matrix contains a cocktail of bioactive molecules that once incorporated within dentin matrix during odontogenesis, these molecules become “fossilized” and retain their biological activity [30, 31]. A variety of growth

factors, such as TGF, VEGF, IGF, have been identified in dentin, but members of TGF-β have been implicated in signalling odontoblast differentiation during tooth development [32]. The tubular structure of dentin confers significant permeability properties on the tissue [33], and demineralization caused by carious process and dentin matrix degradation products may diffuse through the dentinal tubules and cause cellular responses. This involves a sequence of cellular events including cell recruitment, cytodifferentiation, and subsequent activation or up-regulation of the secretory activity of the cells, producing mineralized matrix [34].

In the present study it was used EDTA to mobilize the bioactive protein from dentin. Studies have shown that EDTA can successfully retrieve growth factors from dentin and make them more available for induction of differentiation of dental pulp cells [35, 36]. More recently, it was discovered that dentin extracts after EDTA treatment induce differentiation of dental pulp stem cells (Liu *et al*., 2005). However, the isolate effect of EDTA-soluble dentin matrix on progenitor dental pulp cells differentiation and mineralization remains unclear once the cells were cultured with a mineralization supplement (ascorbic acid, β-glycerophosphate) associated with dentin extracts.

To evaluate the effect of dentin-derived soluble BMPs on dental pulp stem cell differentiation we used the tooth-slice model that has already been used to study the human dental pulp angiogenesis [37]. In a time course assay (unpublished data) the stem cells from human exfoliated teeth seeded into tooth-slice scaffolds EDTA treated started to express the odontoblast differentiation markers (DSPP, DMP-1 and MEPE) after 14 days in culture (*in vitro*). It is import highlight that no mineralization supplement, that induces stem cell differentiation, was used in the culture medium.

In the present study we used the same tooth-slice scaffold model to culture SHED at the presence of neutralized antibodies in the culture medium. The purpose of using these systems was to neutralize the effect of dentin-derived soluble BMPs molecules that are involved in dental pulp stem cell differentiation. The RT-PCR analysis showed no expression of DSPP, DMP- and MEPE markers when BMP-2/4Ab was added to the culture medium. However, in the BMP-7Ab group, the expression of these markers was detected and was shown to follow the pattern presented by SHED cultured in the tooth-slice scaffolds treated with EDTA (without antibodies). No expression of differentiation markers was found for the group where cells were seeded and cultured into PLLA sponge scaffolds. The results suggest that the dentin-derived morphogenic signals BMP-2 and BMP-4 play an important role in dental pulp stem cell differentiation. It was the first

to use the tooth-slice scaffold model to demonstrate the effect of dentin-derived growth factors on dental pulp stem cell differentiation.

Conclusions

- The dental pulp cells (SHED, DPSC and fibroblast) characterized by western blot expressed the BMPs receptors;
- The treatment of SHED by rhBMP-2, -4 and -7 promoted the SHED differentiation;
- BMP-2/4Ab prevented the differentiation markers expression and no inhibitory effect was detected when SHED were cultured in presence of BMP-7Ab.

References

1. Tziafas D. Basic mechanisms of cytodifferentiation and dentinogenesis during dental pulp repair. Int J Dev Biol. 1995 Feb;39(1):281-90.

2. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000 Dec 5;97(25):13625-30.

3. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. Stem cell properties of human dental pulp stem cells. J Dent Res. 2002 Aug;81(8):531-5.

4. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A. 2003 May 13;100(10):5807-12.

5. Smith AJ, Cassidy N, Perry H, Bègue-Kirn C, Ruch JV, Lesot H. Reactionary dentinogenesis. Int J Dev Biol. 1995 Feb;39(1):273-80.

6. About I, Bottero MJ, de Denato P, Camps J, Franquin JC, Mitsiadis TA. Human dentin production in vitro. Exp Cell Res. 2000 Jul 10;258(1):33-41.

7. Rutherford RB, Wahle J, Tucker M, Rueger D, Charette M. Induction of reparative dentine formation in monkeys by recombinant human osteogenic protein-1. Arch Oral Biol. 1993 Jul;38(7):571-6.

8. Nakashima M. Induction of dentine in amputated pulp of dogs by recombinant human bone morphogenetic proteins-2 and -4 with collagen matrix. Arch Oral Biol. 1994 Dec;39(12):1085-9.

9. Six N, Lasfargues JJ, Goldberg M. Differential repair responses in the coronal and radicular areas of the exposed rat molar pulp induced by recombinant human bone morphogenetic protein 7 (osteogenic protein 1). Arch Oral Biol. 2002 Mar;47(3):177-87.

10. Thesleff I, Sharpe P. Signalling networks regulating dental development. Mech Dev. 1997 Oct;67(2):111-23.

11. Jepsen S, Albers HK, Fleiner B, Tucker M, Rueger D. Recombinant human osteogenic protein-1 induces dentin formation: an experimental study in miniature swine. J Endod. 1997 Jun;23(6):378-82.

12. Liu H, Li W, Shi S, Habelitz S, Gao C, Denbesten P. MEPE is downregulated as dental pulp stem cells differentiate. Arch Oral Biol. 2005 Nov;50(11):923-8.

13. Cox CF, Suzuki S. Re-evaluating pulp protection: calcium hydroxide liners vs. cohesive hybridization. J Am Dent Assoc. 1994 Jul;125(7):823-31.

14. Nakashima M, Reddi AH. The application of bone morphogenetic proteins to dental tissue engineering. Nat Biotechnol. 2003 Sep;21(9):1025-32.

15. Decup F, Six N, Palmier B, Buch D, Lasfargues JJ, Salih E, Goldberg M. Bone sialoprotein-induced reparative dentinogenesis in the pulp of rat's molar. Clin Oral Investig. 2000 Jun;4(2):110-9.

16. Goldberg M, Six N, Decup F, Buch D, Soheili Majd E, Lasfargues JJ, Salih E, Stanislawski L. Application of bioactive molecules in pulp-capping situations. Adv Dent Res. 2001 Aug;15(1):91-5.

17. Ritchie HH, Hou H, Veis A, Butler WT. Cloning and sequence determination of rat dentin sialoprotein, a novel dentin protein. J Biol Chem. 1994 Feb 4;269(5):3698-702.

18. MacDougall M, Simmons D, Luan X, Nydegger J, Feng J, Gu TT. Dentin phosphoprotein and dentin sialoprotein are cleavage products expressed from a single transcript coded by a gene on human chromosome 4. Dentin phosphoprotein DNA sequence determination. J Biol Chem. 1997 Jan 10;272(2):835-42.

19. Ritchie HH, Berry JE, Somerman MJ, Hanks CT, Bronckers AL, Hotton D, Papagerakis P, Berdal A, Butler WT. Dentin sialoprotein (DSP) transcripts: developmentally-sustained expression in odontoblasts and transient expression in pre-ameloblasts. Eur J Oral Sci. 1997 Oct;105(5 Pt 1):405-13.

20. Hanks CT, Fang D, Sun Z, Edwards CA, Butler WT. Dentin-specific proteins in MDPC-23 cell line. Eur J Oral Sci. 1998 Jan;106 Suppl 1:260-6.

21. Nakao K, Itoh M, Tomita Y, Tomooka Y, Tsuji T. FGF-2 potently induces both proliferation and DSP expression in collagen type I gel cultures of adult incisor immature pulp cells. Biochem Biophys Res Commun. 2004 Dec 17;325(3):1052-9.

22. Batouli S, Miura M, Brahim J, Tsutsui TW, Fisher LW, Gronthos S, Robey PG, Shi S. Comparison of stem-cell-mediated osteogenesis and dentinogenesis. J Dent Res. 2003 Dec;82(12):976-81.

23. Qin C, Brunn JC, Cadena E, Ridall A, Tsujigiwa H, Nagatsuka H, Nagai N, Butler WT. The expression of dentin sialophosphoprotein gene in bone. J Dent Res. 2002 Jun;81(6):392-4.

24. Bègue-Kirn C, Krebsbach PH, Bartlett JD, Butler WT. Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. Eur J Oral Sci. 1998 Oct;106(5):963-70.

25. Bleicher F, Couble ML, Farges JC, Couble P, Magloire H. Sequential expression of matrix protein genes in developing rat teeth. Matrix Biol. 1999 Apr;18(2):133-43.

26. D'Souza RN, Cavender A, Sunavala G, Alvarez J, Ohshima T, Kulkarni AB, MacDougall M. Gene expression patterns of murine dentin matrix protein 1 (Dmp1) and dentin sialophosphoprotein (DSPP) suggest distinct developmental functions in vivo. J Bone Miner Res. 1997 Dec;12(12):2040-9.

27. Petersen DN, Tkalcevic GT, Mansolf AL, Rivera-Gonzalez R, Brown TA. Identification of osteoblast/osteocyte factor 45 (OF45), a bone-specific cDNA encoding an RGD-containing protein that is highly expressed in osteoblasts and osteocytes. J Biol Chem. 2000 Nov 17;275(46):36172-80.

28. Zhang GX, Mizuno M, Tsuji K, Tamura M. Regulation of mRNA expression of matrix extracellular phosphoglycoprotein (MEPE)/ osteoblast/osteocyte factor 45 (OF45) by fibroblast growth factor 2 in cultures of rat bone marrow-derived osteoblastic cells. Endocrine. 2004 Jun;24(1):15-24.

29. Wei X, Ling J, Wu L, Liu L, Xiao Y. Expression of mineralization markers in dental pulp cells. J Endod. 2007 Jun;33(6):703-8.

30. Finkelman RD, Mohan S, Jennings JC, Taylor AK, Jepsen S, Baylink DJ. Quantitation of growth factors IGF-I, SGF/IGF-II, and TGF-beta in human dentin. J Bone Miner Res. 1990 Jul;5(7):717-23.

31. Ruch JV, Lesot H, Bègue-Kirn C. Odontoblast differentiation. Int J Dev Biol. 1995 Feb;39(1):51-68.

32. Bègue-Kirn C, Smith AJ, Loriot M, Kupferle C, Ruch JV, Lesot H. Comparative analysis of TGF beta s, BMPs, IGF1, msxs, fibronectin, osteonectin and bone sialoprotein gene expression during normal and in vitro-induced odontoblast differentiation. Int J Dev Biol. 1994 Sep;38(3):405-20.

33. Pashley DH, Pashley EL, Carvalho RM, Tay FR. The effects of dentin permeability on restorative dentistry. Dent Clin North Am. 2002 Apr;46(2):211-45.

34. Smith AJ, Tobias RS, Cassidy N, Bégue-Kirn C, Ruch JV, Lesot H. Influence of substrate nature and immobilization of implanted dentin matrix components during induction of reparative dentinogenesis. Connect Tissue Res. 1995;32(1-4):291-6.

35. Roberts-Clark DJ, Smith AJ. Angiogenic growth factors in human dentine matrix. Arch Oral Biol. 2000 Nov;45(11):1013-6.

36. Graham L, Cooper PR, Cassidy N, Nor JE, Sloan AJ, Smith AJ. The effect of calcium hydroxide on solubilisation of bio-active dentine matrix components. Biomaterials. 2006 May;27(14):2865-73.

37. Gonçalves SB, Dong Z, Bramante CM, Holland GR, Smith AJ, Nör JE. Tooth slice-based models for the study of human dental pulp angiogenesis. J Endod. 2007 Jul;33(7):811-4.

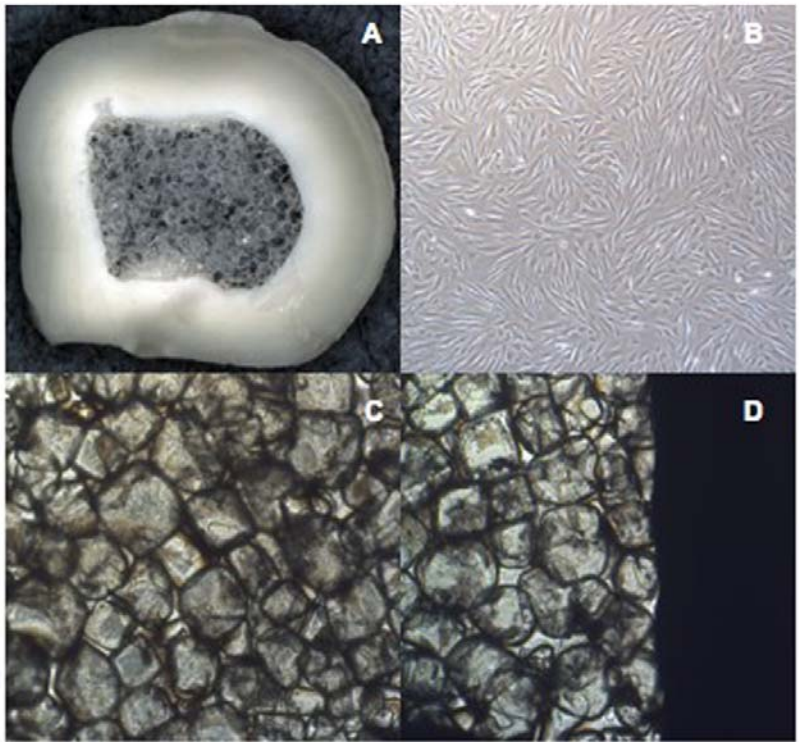

Figure 1. Tooth-slice scaffold model to study the effect of dentin derived morphogens on dental pulp stem cell differentiation. (A) PLLA scaffolds were built inside the pulp chamber of dentin slices obtained from extracted 3rd molars. (B) SHED were cultured in DMEM low glucose (supplemented by 10% FBS and 1% antibiotics) and seeded ($5x10^5$ cells/scaffold) into sponge scaffolds (C) or tooth-slices scaffolds (D).

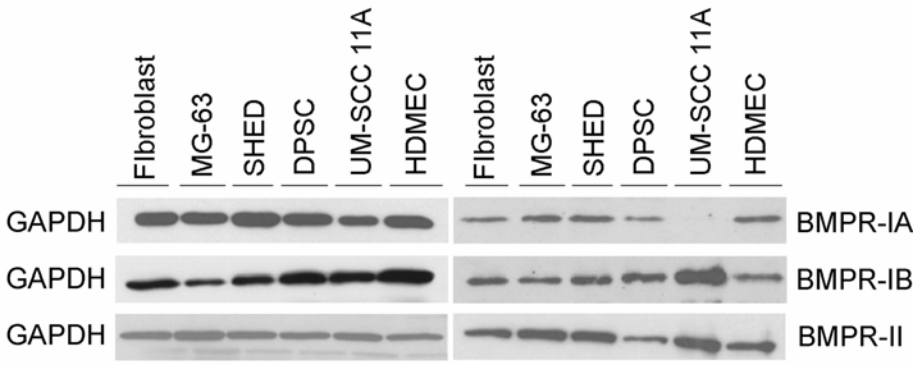

Figure 2. Western blot of BMP receptors expressed by dental pulp cells. SHED, DPSC and fibroblast expressed the BMP-IA, BMP-IB and BMP-II receptors. MG-63, UM-SCC 11A and HDMEC were used as controls.

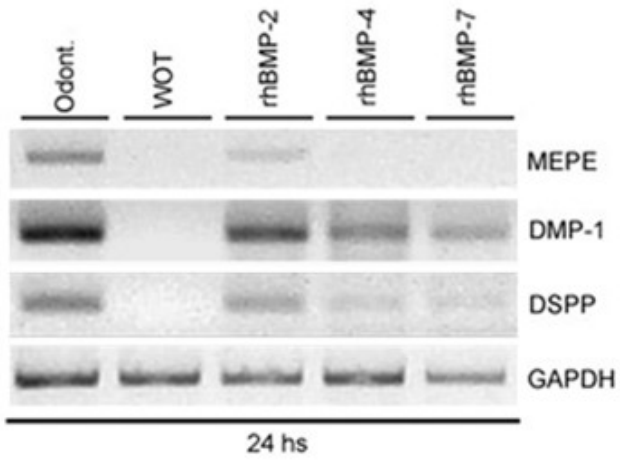

Figure 3. RT-PCR analysis of rhBMP effects on SHED differentiation. Cells were cultured for 24hs in the presence of recombinant rhBMP-2, rhBMP-4 and rhBMP-7 (100ng/mL). Expression of DSPP, DMP-1 and MEPE was found for rhBMP-2, and DSPP and DMP-1 for rhBMP-4 and rhBMP-7 treated SHED, but not for untreated cells (WO-T).

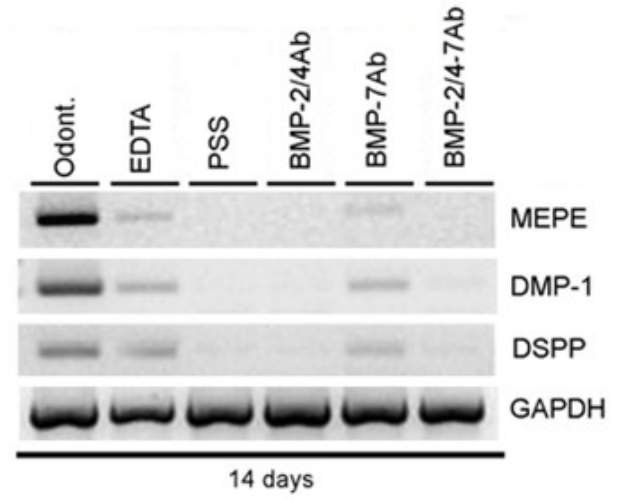

Figure 4. RT-PCR of SHED cultured in tooth-slice scaffolds treated with BMPs neutralizing antibodies. Cells were cultured for 14 days in the presence of neutralizing antibodies BMP-2/4Ab, BMP-7Ab or BMP-2/4Ab and BMP-7Ab (10μg/mL). No expression of odontoblast differentiation markers (DSPP, DMP-1 and MEPE) was found when BMP-2/4Ab was added to the culture medium. However, in the BMP-7Ab group, the expression of these markers followed the differentiation pattern presented by SHED cultured in the tooth-slice scaffolds treated with EDTA.

5. CONSIDERAÇÕES FINAIS

No presente trabalho, princípios de engenharia tecidual foram empregados para o estudo da diferenciação das SHED em células tipo-odontoblásticas. Para permitir a semeadura e o cultivo celular, uma matriz polimérica biodegradável foi construída dentro da cavidade pulpar de fatias dentais humanas. Essa matriz a base de ácido poli-L-lático (PLLA) proporcionou um ambiente favorável à proliferação e diferenciação celular. Um modelo utilizando fatia-dental demonstrou ser adequado para o estudo da angiogênese da polpa dental (GONÇALVES *et al*., 2007).

No modelo fatia-dental/matriz-polimérica, as moléculas bioativas responsáveis pela indução da diferenciação celular foram fornecidas pela matriz dentinária, reconhecida como um "reservatório" de fatores de crescimento (FINKELMAN *et al*., 1990; RUCH *et al*., 1995). O EDTA tem sido utilizado para mobilizar os fatores de crescimento de matrizes mineralizadas (GRAHAM *et al*., 2006). No presente estudo o emprego do EDTA demonstrou ser um bom solubilizador de moléculas bioativas dentinárias.

A RT-PCR revelou que as SHED alteraram sua expressão genética durante o curso do tempo (7, 14, 21 e 28 dias). As células que foram cultivadas *in vitro*, em fatias dentais tratadas com EDTA ou, mesmo sem tratamento, começaram a expressar os marcadores de diferenciação odontoblástica (DSPP, DMP-1 e MEPE) após 14 dias, mantendo esse padrão durante o período experimental (21 e 28 dias). De acordo com Beltz, Torabinejad e Pouresmail (2003), 5.25% de solução de NaOCl foi capaz de dissolver completamente o

conteúdo orgânico dentinário. No grupo onde foi utilizado o NaOCl para remoção dos componentes orgânicos dentinários, ou quando apenas a matriz polimérica sem a fatia dental foi utilizada, não foi observado a expressão dos marcadores de diferenciação.

Os resultados sugerem que a presença da dentina seja suficiente para promover um microambiente favorável à diferenciação celular. Outro fator que pode ter contribuído para esse processo, nos grupos onde a dentina não recebeu tratamento, é a composição de natureza ácida da matriz polimérica utilizada no interior da cavidade pulpar das fatias dentais. Essas estruturas poliméricas (PLLA) liberam ácido como produto de sua degradação, o que pode ter promovido um condicionamento da pré-dentina, mobilizando os fatores de crescimento dentinário.

Avaliando o ensaio de proliferação celular (WST-1), as células cultivadas em matrizes poliméricas onde as fatias dentais receberam o tratamento de EDTA, ou mesmo quando a dentina permaneceu sem tratamento, demonstraram a redução do índice de proliferação a partir dos 14 dias, quando comparados com os grupos onde a dentina foi tratada com NaOCl ou quando foi utilizado a matriz polimérica sem a fatia dental. Os resultados do estudo de proliferação complementam os de diferenciação, pois sustentam a hipótese de que a partir da diferenciação (expressão dos marcadores de diferenciação), as células cessam a divisão.

Segundo Nakashima e Reddi (2003), as proteínas morfogenéticas ósseas (BMPs) são suficientes para promoverem a regeneração dos tecidos dentais,

embora quatro grupos distintos de fatores de crescimento estejam envolvidos com o desenvolvimento crânio-facial.

Para verificar a influência das BMPs dentinárias sobre a diferenciação das SHED, o modelo fatia-dental/matriz-polimérica foi associado com anticorpos específicos para BMPs. O propósito da utilização de anticorpos de neutralização no meio de cultura foi de bloquear e identificar o efeito das BMPs dentinárias sobre as SHED.

A análise da expressão dos marcadores de diferenciação, (DSPP, DMP-1 e MEPE) após 14 dias em cultura *in vitro*, demonstrou que o BMP-2/4Ab exerceu um efeito inibitório quando adicionado ao meio de cultura, pois nenhuma expressão dos marcadores de diferenciação foi identificada. Entretanto, na presença de BMP-7Ab, as células passaram a expressar DSPP, DMP-1 e MEPE, apresentando um padrão de expressão semelhante ao grupo onde as fatias-dentais/matrizes-poliméricas foram tratadas com EDTA. Os resultados sugerem que as proteínas morfogenéticas ósseas BMP-2 e BMP-4 têm um papel mais importante na diferenciação de células-tronco pulpares ou, estão em maiores concentrações na matriz dentinária que a BMP-7, uma vez que a mesma concentração de anticorpos foi utilizada para o tratamento.

Os resultados do presente estudo demonstraram a viabilidade do cultivo de células-tronco pulpares em matrizes poliméricas construídas no interior da cavidade pulpar de fatias dentais. O modelo fatia dental/matriz polimérica proporcionou um microambiente favorável ao crescimento e diferenciação

celular, constituindo-se em uma ferramenta para futuros estudos de regeneração do órgão pulpar.

6. PERSPECTIVAS

A ocorrência de acidentes envolvendo os elementos dentais é bastante comum na população. Ambas as dentições, decídua e permanente, podem estar envolvidas. As seqüelas de um dente avulsionado ou fraturado é bastante marcante, tanto em nível funcional e estético, como psicológico (BEZERRA e TOLEDO, 2005). O comprometimento pulpar em casos de traumatismo dental pode ser de caráter transitório ou permanente.

A ocorrência de necrose pulpar pode variar de 85-96% em dentes avulsionados e de 70-100% em dentes que sofreram intrusão (ANDREASEN & ANDREASEN, 1994). Apesar das alternativas terapêuticas demonstrarem um bom índice de sucesso clínico e radiográfico, os dentes endodonticamente tratados têm sua resistência mecânica reduzida pelo preparo biomecânico. Dentes permanentes jovens com rizogênese incompleta, que sofreram necrose pulpar, invariavelmente terão sua estrutura fragilizada pela interrupção no processo de dentinogênese. O tratamento disponível atualmente, baseado em trocas de hidróxido de cálcio e preenchimento do canal endodôntico com guta-percha, elimina a infecção bacteriana, mas não permite a formação radicular completa. Dessa forma, a ocorrência de traumas secundários pode resultar em fratura radicular com necessidade de exodontia.

A partir da descoberta que células-tronco de alta qualidade podem ser

extraídas da polpa dental, inclusive de dentes decíduos esfoliados, aumentaram as possibilidades para a regeneração do complexo dentino-pulpar em dentes endodonticamente comprometidos.

Sob perspectivas clínicas, a transição da dentição decídua para permanente é um evento fisiológico único onde a erupção dos dentes permanentes ocorre coordenadamente com a reabsorção das raízes dos dentes decíduos (MIURA *et al*., 2003). A fase da dentição mista ocorre em crianças de 6 aos 12 anos de idade. Nesse período, a prevalência de traumas é alta, e no caso de uma avulsão com conseqüências pulpares, a situação do remanescente dental seria mais favorável para a regeneração do órgão pulpar, pois o ápice dental ainda se encontra com uma abertura foramidal favorável à revascularização (ROBERTS & LONGHURST, 1996), além do paciente possuir dentes decíduos em processo de esfoliação, potenciais doadores de células-tronco para a terapia celular.

Com os avanços científicos referentes à biologia molecular e celular, bem como na área da engenharia tecidual, talvez em um futuro não muito distante, a regeneração do órgão pulpar em dentes permanentes jovens seja uma realidade clínica, onde células associadas a matrizes biodegradáveis e fatores de crescimento serão inseridos no interior do conduto endodôntico para o restabelecimento da vitalidade, função e estética.

7. CONCLUSÕES

- Matrizes poliméricas confeccionadas no interior da cavidade pulpar de fatias dentais de dentes permanentes promoveram um microambiente favorável para o crescimento e diferenciação das SHED;
- A RT-PCR demonstrou que as SHED alteraram sua expressão genética durante o curso do tempo quando cultivadas *in vitro* e *in vivo* em matrizes poliméricas no interior de fatias dentais;
- O índice de proliferação celular foi influenciado pelo tipo tratamento dentinário, diminuindo com a diferenciação celular;
- Os sinais morfogenéticos dentinários (BMP-2/4), no modelo fatia-dental/matriz-polimérica, desempenham um papel importante na diferenciação de células tronco pulpares.

8. REFERÊNCIAS

ANDERSON, D.J.; GAGE, F.H.; WEISSMAN, I.L. Can stem cells cross lineage boundaries? **Nat Med**, New York, v.7, n.4, p.393-5 Apr. 2001.

ANDREASEN, J.; ANDREASEN, F. **Textbook and color atlas of traumatic injuries to the teeth:** Iowa State Press. 1994. 776 p.

BARRETT, J.; MCCARTHY, D. Bone marrow transplantation for genetic disorders. **Blood Rev**, Edinburgh, v.4, n.2, p.116-31 Jun. 1990.

BELTZ, R.E.; TORABINEJAD, M.; POURESMAIL, M. Quantitative analysis of the solubilizing action of MTAD, sodium hypochlorite, and EDTA on bovine pulp and dentin. **J Endod**, Baltimore, v.29, n.5, p.334-7 May. 2003.

BEZERRA, A.; TOLEDO, A. Traumatismos em dentes decíduos. In:TOLEDO, O **Fundamentos para a prática Clínica**. São Paulo: Editorial Premier, 2005. p.267-287.

BJORNSON, C.R.; RIETZE, R.L.; REYNOLDS, B.A.; MAGLI, M.C.; VESCOVI, A.L. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. **Science**, Washington, v.283, n.5401, p.534-7 Jan 22. 1999.

BUCAL, C.N.D.S. Projeto SB Brasil 2003 Condições de saúde bucal da população brasileira 2002-2003. SAÚDE, MD**:** 51 p. 2004.

DECUP, F.; SIX, N.; PALMIER, B.; BUCH, D.; LASFARGUES, J.J.; SALIH, E.*, et al.* Bone sialoprotein-induced reparative dentinogenesis in the pulp of rat's molar. **Clin Oral Investig**, Berlim, v.4, n.2, p.110-9 Jun. 2000.

FINKELMAN, R.D.; MOHAN, S.; JENNINGS, J.C.; TAYLOR, A.K.; JEPSEN, S.; BAYLINK, D.J. Quantitation of growth factors IGF-I, SGF/IGF-II, and TGF-beta in human dentin. **J Bone Miner Res**, Washington, v.5, n.7, p.717-23 Jul. 1990.

FORTIER, L.A. Stem cells: classifications, controversies, and clinical applications. **Vet Surg**, Filadélfia, v.34, n.5, p.415-23 Sep-Oct. 2005.

FREED, L.E.; VUNJAK-NOVAKOVIC, G.; BIRON, R.J.; EAGLES, D.B.; LESNOY, D.C.; BARLOW, S.K.*, et al.* Biodegradable polymer scaffolds for tissue engineering. **Biotechnology (N Y)**, v.12, n.7, p.689-93 Jul. 1994.

FUCHS, E.; SEGRE, J.A. Stem cells: a new lease on life. **Cell**, Cambridge, v.100, n.1, p.143-55 Jan 7. 2000.

GARDNER, R.L. Stem cells: potency, plasticity and public perception. **J Anat**, Londres, v.200, n.Pt 3, p.277-82 Mar. 2002.

GOLDBERG, M.; SIX, N.; DECUP, F.; BUCH, D.; SOHEILI MAJD, E.; LASFARGUES, J.J. *et al.* Application of bioactive molecules in pulp-capping situations. **Adv Dent Res**, Washington, v.15, p.91-5 Aug. 2001.

GOLDBERG, M. ; SIX, N. ; DECUP, F. ; LASFARGUES, J.J. ; SALIH, E. ; TOMPKINS, K*, et al.* Bioactive molecules and the future of pulp therapy. **Am J Dent**, Santo Antonio, v.16, n.1, p.66-76 Feb. 2003.

GONCALVES, S.B.; DONG, Z.H.; BRAMANTE, C.M.; HOLLAND, G.R.; SMITH, A.J.; NOR, J.E. Tooth slice-based models for the study of human dental pulp angiogenesis. **Journal of Endodontics**, Baltimore, v.33, n.7, p.811-814. 2007.

GRAHAM, L.; COOPER, P.R.; CASSIDY, N.; NOR, J.E.; SLOAN, A.J.; SMITH, A.J. The effect of calcium hydroxide on solubilisation of bio-active dentine matrix components. **Biomaterials**, Guilford, v.27, n.14, p.2865-73 May. 2006.

GRONTHOS, S.; BRAHIM, J.; LI, W.; FISHER, L.W.; CHERMAN, N.; BOYDE, A. *et al.* Stem cell properties of human dental pulp stem cells. **J Dent Res**, Chicago, v.81, n.8, p.531-5 Aug. 2002.

GRONTHOS, S.; MANKANI, M.; BRAHIM, J.; ROBEY, P.G.; SHI, S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. **Proc Natl Acad Sci U S A**, Washington, v.97, n.25, p.13625-30 Dec 5. 2000.

GU, K.; SMOKE, R.H.; RUTHERFORD, R.B. Expression of genes for bone morphogenetic proteins and receptors in human dental pulp. **Arch Oral Biol**, Oxford, v.41, n.10, p.919-23 Oct. 1996.

HARADA, H.; KETTUNEN, P.; JUNG, H.S.; MUSTONEN, T.; WANG, Y.A.; THESLEFF, I. Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. **J Cell Biol**, Nova York, v.147, n.1, p.105-20 Oct 4. 1999.

HOHLFELD, J.; DE BUYS ROESSINGH, A.; HIRT-BURRI, N.; CHAUBERT, P.; GERBER, S.; SCALETTA, C*, et al.* Tissue engineered fetal skin constructs for paediatric burns. **Lancet**, Londres, v.366, n.9488, p.840-2 Sep 3-9. 2005.

JADLOWIEC, J.A.; CELIL, A.B.; HOLLINGER, J.O. Bone tissue engineering: recent advances and promising therapeutic agents. **Expert Opin Biol Ther**, Londres, v.3, n.3, p.409-23 Jun. 2003.

KENNY, A.B.; HITZIG, W.H. Bone marrow transplantation for severe combined immunodeficiency disease. Reported from 1968 to 1977. **Eur J Pediatr**, Nova York, v.131, n.3, p.155-77 Jun 28. 1979.

LANGER, R.; VACANTI, J.P. Tissue engineering. **Science**, Washington, v.260, n.5110, p.920-6 May 14. 1993.

LIU, J.; JIN, T.; RITCHIE, H.H.; SMITH, A.J.; CLARKSON, B.H. In vitro differentiation and mineralization of human dental pulp cells induced by dentin extract. **In Vitro Cell Dev Biol Anim**, Columbia, v.41, n.7, p.232-8 Jul-Aug. 2005.

MARTIN-RENDON, E.; WATT, S.M. Exploitation of stem cell plasticity. **Transfus Med**, Oxford, v.13, n.6, p.325-49 Dec. 2003.

MARTIN, I.; PADERA, R.F.; VUNJAK-NOVAKOVIC, G.; FREED, L.E. In vitro differentiation of chick embryo bone marrow stromal cells into cartilaginous and bone-like tissues. **J Orthop Res**, Nova York, v.16, n.2, p.181-9 Mar. 1998.

MIURA, M.; GRONTHOS, S.; ZHAO, M.; LU, B.; FISHER, L.W.; ROBEY, P.G. *et al.* SHED: stem cells from human exfoliated deciduous teeth. **Proc Natl Acad Sci U S A**, Washington, v.100, n.10, p.5807-12 May 13. 2003.

MURRAY, P.E.; SMITH, A.J. Saving pulps: a biological basis. An overview. **Prim Dent Care**, London, v. 9, n.1, p. 21–6, Jan 2002.

NAKASHIMA, M. Induction of dentine in amputated pulp of dogs by recombinant human bone morphogenetic proteins-2 and -4 with collagen matrix. **Arch Oral Biol**, Oxford, v.39, n.12, p.1085-9 Dec. 1994.

NAKASHIMA, M. Tissue engineering in endodontics. **Aust Endod J**, Melbourne, v.31, n.3, p.111-3 Dec. 2005.

NAKASHIMA, M.; AKAMINE, A. The application of tissue engineering to regeneration of pulp and dentin in endodontics. **J Endod**, Baltimore, v.31, n.10, p.711-8 Oct. 2005.

NAKASHIMA, M.; NAGASAWA, H.; YAMADA, Y.; REDDI, A.H. Regulatory role of transforming growth factor-beta, bone morphogenetic protein-2, and protein-4 on gene expression of extracellular matrix proteins and differentiation of dental pulp cells. **Dev Biol**, Nova York, v.162, n.1, p.18-28 Mar. 1994.

NAKASHIMA, M.; REDDI, A.H. The application of bone morphogenetic proteins to dental tissue engineering. **Nat Biotechnol**, Nova York, v.21, n.9, p.1025-32 Sep. 2003.

PASHLEY, D.H.; PASHLEY, E.L.; CARVALHO, R.M.; TAY, F.R. The effects of dentin permeability on restorative dentistry. **Dent Clin North Am**, Filadélfia, v.46, n.2, p.211-45, v-vi Apr. 2002.

RAMOSHEBI, L.N.; MATSABA, T.N.; TEARE, J.; RENTON, L.; PATTON, J.; RIPAMONTI, U. Tissue engineering: TGF-beta superfamily members and delivery systems in bone regeneration. **Expert Rev Mol Med**, Cambridge, v.4, n.20, p.1-11 Sep. 2002.

RAO, M.S. Stem sense: a proposal for the classification of stem cells. **Stem Cells Dev**, Larchmont, v.13, n.5, p.452-5 Oct. 2004.

ROBERTS, G.; LONGHURST, P. **Oral and dental trauma in children and adolescents**: Oxford University Press. 1996

RUCH, J.V.; LESOT, H.; BÈGUE-KIRN, C. Odontoblast differentiation. **Int J Dev Biol**, Vizcaya, v. 39, n. 1, p. 51-68, Feb. 1995.

SACHLOS, E.; CZERNUSZKA, J.T. Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. **Eur Cell Mater**, Glasgow, v.5, p.29-39; discussion 39-40 Jun 30. 2003.

SHI, S.; GRONTHOS, S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. **J Bone Miner Res**, Washington, v.18, n.4, p.696-704 Apr. 2003.

SHI, S.; ROBEY, P.G.; GRONTHOS, S. Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis. **Bone**, Elmsford, v.29, n.6, p.532-9 Dec. 2001.

SIX, N.; LASFARGUES, J.J.; GOLDBERG, M. Differential repair responses in the coronal and radicular areas of the exposed rat molar pulp induced by recombinant human bone morphogenetic protein 7 (osteogenic protein 1). **Arch Oral Biol**, Oxford, v.47, n.3, p.177-87 Mar. 2002.

SMITH, A.; SLOAN, A.; MATTHEWS, J.; MURRAY, P.; LUMLEY, P. Reparative processes in dentine and pulp. In:M, A, G, E*, et al* **Toothwear and sensivity**. London: Martin-Dunitz, p.53-66, 2000.

SMITH, A.G. Embryo-derived stem cells: of mice and men. **Annu Rev Cell Dev Biol**, Palo Alto, v.17, p.435-62. 2001.

SMITH, A.J.; CASSIDY, N.; PERRY, H.; BEGUE-KIRN, C.; RUCH, J.V.; LESOT, H. Reactionary dentinogenesis. **Int J Dev Biol**, Vizcaya, v.39, n.1, p.273-80 Feb. 1995.

STEVENS, M.M.; MARINI, R.P.; SCHAEFER, D.; ARONSON, J.; LANGER, R.; SHASTRI, V.P. In vivo engineering of organs: the bone bioreactor. **Proc Natl Acad Sci U S A**, Washington, v.102, n.32, p.11450-5 Aug 9. 2005.

TAYLOR, M.S.; DANIELS, A.U.; ANDRIANO, K.P.; HELLER, J. Six bioabsorbable polymers: in vitro acute toxicity of accumulated degradation products. **J Appl Biomater**, Nova York, v.5, n.2, p.151-7 Summer. 1994.

THESLEFF, I.; SHARPE, P. Signalling networks regulating dental development. **Mech Dev**, Shannon, v.67, n.2, p.111-23 Oct. 1997.

TZIAFAS, D. Basic mechanisms of cytodifferentiation and dentinogenesis during dental pulp repair. **Int J Dev Biol**, Vizcaya, v.39, n.1, p.281-90 Feb. 1995.

TZIAFAS, D. The future role of a molecular approach to pulp-dentinal regeneration. **Caries Res**, Basel, v.38, n.3, p.314-20 May-Jun. 2004.

VASITA, R.; KATTI, D.S. Growth factor-delivery systems for tissue engineering: a materials perspective. **Expert Rev Med Devices**, Londres, v.3, n.1, p.29-47 Jan. 2006.

VASSILOPOULOS, G.; RUSSELL, D.W. Cell fusion: an alternative to stem cell plasticity and its therapeutic implications. **Curr Opin Genet Dev**, Londres, v.13, n.5, p.480-5 Oct. 2003.

WAGERS, A.J.; WEISSMAN, I.L. Plasticity of adult stem cells. **Cell**, Cambridge, v.116, n.5, p.639-48 Mar 5. 2004.

WARNKE, P.H.; SPRINGER, I.N.; WILTFANG, J.; ACIL, Y.; EUFINGER, H.; WEHMOLLER, M. *et al.* Growth and transplantation of a custom vascularised bone graft in a man. **Lancet**, Londres, v.364, n.9436, p.766-70 Aug 28-Sep 3. 2004.

WINGARD, J.; DEMETRI, G. **Clinical applications of cytokines and growth factors**: Springer. 1999

YOUNG, C.S.; TERADA, S.; VACANTI, J.P.; HONDA, M.; BARTLETT, J.D.; YELICK, P.C. Tissue engineering of complex tooth structures on biodegradable polymer scaffolds. **J Dent Res**, Chicago, v.81, n.10, p.695-700 Oct. 2002.