THAISÂNGELA RODRIGUES LOPES E SILVA GOMES
CIRURGIÃ-DENTISTA

ANÁLISE DA REGULAÇÃO DE GENES ASSOCIADOS AO METABOLISMO DO FOSFATO EM CÉLULAS DA POLPA E LIGAMENTO PERIODONTAL E SUAS ASSOCIAÇÕES NA REGENERAÇÃO PERIODONTAL. ESTUDO "IN VITRO" E "IN VIVO".

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de Doutor em Clínica Odontológica, Área de Periodontia.

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PIRACICABA-SP
2011

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FACULDADE DE ODONTOLOGIA DE PIRACICABA
Bibliotecária: Elis Regina Alves dos Santos – CRB-8ª / 8099

R618a

Rodrigues, Thaisângela.
Análise da regulação de genes associados ao metabolismo do fosfato em células da polpa e ligamento periodontal e suas associações na regeneração periodontal: estudo "in vitro" e "in vivo" / Thaisângela Rodrigues Lopes e Silva Gomes. -- Piracicaba, SP: [s.n.], 2011.

Orientadores: Francisco Humberto Nociti Júnior, Karina Gonzales Silvério Ruiz.
Tese (Doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Hipofosfatasia. 2. Cultura celular. 3. Cemento dentário. 4. Fosfatase alcalina. I. Nociti Júnior, Francisco Humberto. II. Ruiz, Karina Gonzales Silvério. III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

(eras/fop)

Título em Inglês: Analysis of phosphate regulation genes in pulp and periodontal ligament cells and their associations with periodontal regeneration: study "in vitro" and "in vivo"
Palavras-chave em Inglês (Keywords): 1. Hypophosphatasia. 2. Cell culture. 3. Dental cementum. 4. Alkaline phosphatase
Área de Concentração: Periodontia
Titulação: Doutor em Clínica Odontológica
Banca Examinadora: Karina Gonzales Silvério Ruiz, Mario Taba Júnior, Carlos Rossa Júnior, Enilson Antônio Sallum, Bruna Rabelo Amorim
Data da Defesa: 24-02-2011
Programa de Pós-Graduação em Clínica Odontológica

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 24 de Fevereiro de 2011, considerou a candidata THAISÂNGELA RODRIGUES LOPES E SILVA GOMES aprovada.

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_____*DEDICATÓRIA*

Dedico este trabalho

Aos meus pais, ***Anadir*** *e* ***Edilamar****,*

pessoas essenciais na minha vida, fonte de amor e sabedoria.

Ao meu querido marido, ***Ricardo****,*

exemplo de honestidade e determinação,

sempre me motivando e me alimentando com seu amor,

carinho e paciência.

E aos meus irmãos, ***Elisângela****,* ***Mariângela*** *e* ***Marlos****,*

meus grandes amigos,

que me proporcionaram alegria durante esse período,

aliviando a saudade provocada pela distância.

AGRADECIMENTOS

À **Deus**, essência da sabedoria e da ciência. Minha fonte de amor e paz interior.

À Universidade Estadual de Campinas, na pessoa do Magnífico Reitor **Prof. Dr. Fernando Ferreira Costa.**

À Direção da Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, nas pessoas do Diretor **Prof. Dr. Jacks Jorge Junior** e do Diretor Associado **Prof. Dr. Alexandre Augusto Zaia.**

À **Prof[a]. Dr[a]. Renata Cunha Matheus Rodrigues Garcia**, coordenadora dos Cursos de Pós-Graduação da FOP/UNICAMP, e ao **Prof. Dr. Márcio de Moraes**, Coordenador do curso de Pós-Graduação em Clínica Odontológica da FOP/UNICAMP.

À **FAPESP** pelo apoio ao trabalho em forma de auxílio à pesquisa e bolsa de doutorado.

Ao **Prof. Dr. Francisco Humberto Nociti Jr.,** meu orientador. Chico, muito obrigada por toda confiança, responsabilidades e oportunidades oferecidas a mim, o que fizeram com que eu crescesse como pessoa e como pesquisadora. Obrigada por passar o seu amor e dedicação à pesquisa de qualidade, e por sempre me motivar a buscar o melhor de mim.

À **Prof[a]. Dr[a]. Karina Gonzales Silvério Ruiz**, pelos momentos que passamos durante as atividades de laboratório, compartilhando erros e acertos, procurando sempre o aprimoramento de nossos conhecimentos. Obrigada por ser uma grande amiga!

Aos professores da área: **Prof. Dr. Antônio Wilson Sallum**, **Prof. Dr. Sérgio de Toledo**, **Prof. Dr. Enilson Antônio Sallum**, **Prof. Dr. Francisco Humberto Nociti Jr.**, **Prof. Dr.**

Márcio Zaffalon Casati e a **Prof[a]. Dr[a]. Karina Gonzales Silvério Ruiz**, pelos conhecimentos compartilhados, apoio, disponibilidade e atenção dispensada a mim.

Aos professores **Dr. Agenor Montebello Filho,** Departamento de Diagnóstico Oral, área de Radiologia Odontológica, e **Dr. Antônio Fernando Martorelli de Lima** (*in memoriam*), Departamento de Prótese e Periodontia, área de Periodontia, que me iniciaram na vida científica, mostrando quão fascinante é a ciência.

À **Prof[a]. Dr[a]. Martha Somerman** e ao **Dr. Brian Foster**, da Universidade de Washington, por terem me recebido de forma extraordinária em seu laboratório, me dando a oportunidade de aprender novas técnicas, bem como de aprimorar minha capacidade científica através de um convívio próximo e valioso com esses pesquisadores

À **Prof[a]. Dr[a]. Cecília Gatti Guirado**, *in memoriam*, do Departamento de Odontologia Infantil, área de Odontoperiatria, pela condução do tratamento odontopediátrico dos pacientes com hipofosfatasia, e por toda dedicação e carinho que sempre teve com os seus alunos e pacientes.

Ao **Prof. Dr. João Sarmento Pereira Neto**, do Departamento de Odontologia infantil, área de Ortodontia, pela condução do tratamento ortodôntico dos pacientes com hipofosfatasia, com muita cordialidade e atenção para comigo.

Aos professores que participaram da banca de qualificação: **Prof. Dr. Ricardo Della Coletta, Prof[a]. Dr[a]. Daiane Cristina Peruzzo** e **Prof[a]. Dr[a]. Bruna Rabelo Amorim**, por aceitarem prontamente o convite para comporem a banca de qualificação desta tese e pelas contribuições realizadas.

Aos professores que participaram da banca de defesa: **Prof[a]. Dr[a]. Karina Gonzales Silvério Ruiz, Prof. Dr. Mário Taba Jr., Prof. Dr. Carlos Rossa Jr., Prof. Dr. Enilson**

Antônio Sallum e **Prof[a]. Dr[a]. Bruna Rabelo Amorim**, por aceitarem prontamente o convite para comporem a banca de defesa desta tese e pelas contribuições realizadas.

Aos funcionários do Biotério: **Sr. Vanderlei** e **Sr. Fábio** pelo auxílio dispensado durante os períodos experimentais e aos demais funcionários da faculdade, como o **Sr. João eletricista**, que viabilizam nosso trabalho.

Aos **pacientes** desse estudo, que confiaram em nossa capacidade profissional, cooperando com o tratamento e com as tomadas de dados de forma exemplar. Muito obrigada pela dedicação ao tratamento e confiança.

Aos amigos e colegas de turma, **Beatriz de Brito Bezerra**, **Daniela da Silva Feitosa**, **Fabrícia Ferreira Suaid**, **Fernanda Vieira Ribeiro**, **Flávia Sammartino Mariano** (da turma da microbiologia, mas a tenho como da minha turma), **Liana Linhares Lima**, **Ezymar Gomes Cayana**, **Mauro Pedrine Santamaria**, **Marisi Aidar** (da histologia), **Renato Corrêa Viana Casarin** e **Wagner Leal Serra e Silva Filho**. Pelo convívio durante esses anos, que com certeza deu base a grandes amizades. Saibam que cada um de vocês tem um lugar muito especial no meu coração

Aos **Departamentos de Morfologia**, área de Histologia e Embriologia, **e de Diagnóstico Oral**, áreas de Microbiologia e Imunologia e área de Patologia, por estarem sempre de portas abertas aos alunos da Periodontia, compartilhando tanto materiais, estrutura como conhecimentos.

À **Eliete Marin** e à **Regina Caetano**, adoráveis pessoas que sempre estão à disposição da Periodontia. Pela atenção, competência, cordialidade e principalmente, pela amizade que existe entre nós. Muito obrigada!

À **Mariana Fugolin,** minha querida amiga Mari, pelo trabalho no laboratório de histologia, pela sua disponibilidade e atenção para comigo. Obrigada pelo nosso convívio diário cheio de boas conversas e trabalho intenso, que irão deixar saudades.

Aos colegas e amigos do pós-doutorado, **Bruna Rabelo Amorim**, **Cristiane Ribeiro Salmon**, **Luciane Martins e Marcelo Rocha Marques,** pelas trocas de conhecimentos de valor imensurável que fizeram parte de minha formação como pesquisadora, e pela amizade conquistada.

Aos colegas e amigos da Periodontia: **Ana Paula Giorgetti, Ana Regina Oliveira Moreira, Fernanda Velasco, Hugo Felipe do Vale, Lucas Alves Moura, Lucas Araújo Queiróz, Maria Fernanda Santos Peres, Mirella Lindoso G. Campos, Mônica Grazieli Corrêa, Tatiana Meulman e Tiago Taiete**. Obrigada a todos pelos bons momentos que cada um proporcionou de sua forma particular junto a mim durante essa jornada. Saibam que podem contar comigo sempre que precisarem!

Às queridas alunas de iniciação científica, **Mayra Laino Albiero** e **Miki Taketomi Saito**, agora alunas do Mestrado, por serem tão dedicadas e por terem dado a mim a chance de exercer a prática do ensino. Obrigada por compreenderem os meus momentos de dúvidas e dado a oportunidade de eu aprimorar cada vez mais para poder passar algum conhecimento a vocês.

E ao que é de mais importante na minha vida, a minha família!!!
Meus pais **Anadir** e **Edilamar**, e meus irmãos, **Elisângela**, **Mariângela** e **Marlos**. Obrigada por acreditarem em mim dando toda a estrutura emocional para que fosse possível tornar realidade o meu sonho de criança, o de ser professora, e agora pesquisadora.
E, ao meu marido **Ricardo**, muito obrigada por toda sua dedicação, paciência, compreensão e amor. Obrigada por ter dividido comigo os nossos quatro anos de casamento com os quatro anos de doutorado.

_____*EPÍGRAFE*

"Não sei o que o mundo dirá de minha obra. A mim, parece que nunca acabei de ser criança. Uma criança que brincou na praia, que encontrou uma pedra bem polida, uma concha multicolorida, enquanto o grande oceano da verdade continua a se estender, ainda inexplorado, diante de meus olhos."

Isaac Newton.

RESUMO

A formação deficiente do cemento acelular sobre a raiz dentinária de pacientes com hipofosfatasia (HPP) elucida a importância da expressão local da enzima fosfatase alcalina (TNAP) durante a cementogênese. Os objetivos desses estudos foram: i) apresentar dois casos clínicos de perda prematura de dentes decíduos diagnosticados como odonto-HPP; ii) caracterizar diferencialmente o perfil de expressão gênica de células da polpa e ligamento periodontal em indivíduos saudáveis e com HPP em relação a genes envolvidos com o metabolismo do fosfato inorgânico (P_i). iii) caracterizar o reparo e regeneração dos tecidos periodontais em modelo de fenestração periodontal realizado em camundongos com bloqueio do gene da proteína de anquilose (*Ank* KO). **Métodos:** i) Pacientes apresentaram esfoliação precoce dos decíduos aos dois anos de idade, mantendo-se em tratamento odontológico rigoroso. Ambos apresentaram baixos níveis séricos de fosfatase alcalina (ALP), porém sem anormalidade esquelética chegando-se ao diagnóstico de odonto-HPP; ii) Análise da expressão de genes associados à homeostasia entre fosfato e pirofosfato (P_i/PP_i) foi realizada em tecidos da polpa e ligamento periodontal (PDL) de indivíduos saudáveis. Cultura de células primárias da polpa e PDL obtidas de pacientes saudáveis e com HPP foram estabelecidas para os ensaios de mineralização e expressão gênica; iii) Defeitos de fenestração periodontal (2mm/1mm/0,5mm) foram criados na vestibular de molares mandibulares de camundongos *Ank* KO e wild-type (WT). Após 15 e 30 dias das cirurgias, as mandíbulas foram coletadas para análise histológica, histomorfometria, avaliação *in vivo* com marcadores fluorescentes, e imunohitoquímica para proteínas da matriz extracelular. **Resultados:** i) Cuidados odontopediátricos e terapia periodontal de suporte foram realizados durante 19 anos com o objetivo de prevenir/adiar possíveis perdas de dentes permanentes; ii) Nos tecidos saudáveis, PDL manteve maior e significativa expressão basal para os genes reguladores chaves do PP_i quando comparado com a polpa, como fosfatase alcalina (Alpl), proteína de anquilose pregressiva (Ank), e glicoproteína 1 (ectonucleotide pyrophosphatase/phosphodiesterase 1 - Enpp1). *In vitro*, embora as alterações mais dramáticas fossem encontradas nas células do PDL, tanto as células HPP-

PDL como HPP-polpa exibiram significativamente baixa atividade de ALP, menor mineralização e expressões reduzidas dos genes associados com a mineralização e regulação do P_i/PP_i, comparado ao controle; iii) Grande quantidade de novo cemento foi observada nos camundongos *Ank* KO após 15 e 30 dias da cirurgia. ($p<0,05$). Os marcadores fluorescentes indicaram maior atividade de deposição cementária nas áreas dos defeitos nos *Ank* KO vs. WT. Durante os períodos de 15 e 30 dias de cicatrização, regeneração do cemento e células associadas nos *Ank* KO recapitularam o padrão de expressão gênica mapeada durante o desenvolvimento, incluindo expressão limitada de BSP e forte OPN e DMP1 na matriz cementária, bem como elevada expressão de NPP1 nos cementoblastos. **Conclusões:** Dentro dos limites desse estudo, podemos concluir que: i) a perda prematura de dentes decíduos na ausência de desordens esqueléticas pode servir como um sinal inicial crítico para o diagnóstico de odonto-HPP e outros subtipos; ii) os dados sugerem que há uma diferença importante no comportamento *in vitro* entre as células controle e HPP, incluindo a expressão basal dos genes relacionados ao cemento bem como suas capacidades de promoverem a formação de minerais; iii) Dentro dos limites do estudo, os achados sugerem que níveis reduzidos de PP_i local pode promover um aumento da regeneração do cemento. Portanto, a modulação entre P_i/PP_i pode ser uma potente abordagem terapêutica para alcançar melhoras na regeneração do cemento.

Palavras-chave: polpa dental, ligamento periodontal, metabolismo de fosfato, hipofosfatasia, cemento, ANK, regeneração periodontal.

ABSTRACT

The defective formation of acellular cementum along the tooth root in patients with hypophosphatasia (HPP) have been highlighted the importance of local expression of alkaline phosphatase enzyme (TNAP) for cementogenesis. The aims of these studies were: i) to present two clinical cases that premature loss of primary teeth guided to the diagnosis of odontohypophosphatasia (odonto-HPP); ii) to determine factors contributing to the divergent response of the periodontium and dentin to alterations of phosphate (P_i) metabolism; and iii) to analyze tissue repair and regeneration in a periodontal fenestration model in *Ank* knock-out (KO) mice. **Methods:** i) Patients had teeth exfoliation at 2 years-old, and have been under maintenance visits since then. Both exhibited low levels of serum alkaline phosphatase (ALP) activity, but no additional skeletal abnormalities, prompting a diagnosis of odonto-HPP; ii) Constitutive expression of P_i/PP_i-associated genes in periodontal ligament (PDL) versus pulp tissues obtained from healthy subjects were analyzed. Primary cell cultures from control and HPP-PDL and pulp tissues were established to assay mineralization and gene expression; iii) Periodontal fenestration defects (2mm/1mm/0.5mm) were created on the buccal aspects of mandibular molars in *Ank* KO and wild-type (WT) mice. Mandibles were harvested at 15, and 30 days post-surgery for histology, histomorphometry, evaluation of *in vivo* fluorochrome labeling, and immunohistochemistry (IHC) for extracellular matrix proteins. **Results:** i) Pediatric dental care and supportive periodontal therapy were performed during the subsequent 19 years, aimed at avoiding or delaying loss of permanent teeth. ii) In healthy tissues, PDL maintained significantly higher basal expression of key PP_i regulators, liver/bone/kidney alkaline phosphatase (Alpl), progressive ankylosis protein (Ank) and ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1), versus pulp. *In vitro*, although more dramatic changes were found for PDL-harvested cells, both HPP-PDL and HPP-pulp cells exhibited significantly lower alkaline phosphatase activity, mineralization, and depressed expression of genes associated with mineralization and regulation of P_i/PP_i, versus control cells. iii) A greater amount of new cementum was observed for *Ank* KO mice at 15 and 30

days post-surgery ($p<0.05$). Fluorochrome labeling further indicated a higher appositional activity in the defect areas in *Ank* KO vs. controls. At days 15 and 30 during healing, regenerating cementum and associated cells in *Ank* KO recapitulated expression patterns mapped during development, including limited BSP and strong OPN and DMP1 in the cementum matrix, as well as elevated NPP1 in cementoblasts. **Conclusions:** Within the limits of these studies, we can conclude that: i) premature loss of deciduous teeth in absence of skeletal disorders may serve as a critical trigger sign for diagnosis of odonto-HPP or other subtypes; ii) the data suggest that there are important differences in the *in vitro* behavior of control versus HPP cells, including basal expression of cementum-related genes as well as their capacity to promote mineral formation; iii) these findings suggest that reduced local levels of PP_i can promote increased cementum regeneration. Therefore, local modulation of P_i/PP_i may be a potential therapeutic approach for achieving improved cementum regeneration.

Key words: dental pulp, periodontal ligament, phosphate metabolism, hypophosphatasia, cementum, ANK, periodontal regeneration

PREFÁCIO

Esta tese está baseada nos seguintes artigos científicos:

1. Cementum and periodontal defects resulting from odontohypophosphatasia predispose for premature tooth loss. In: McCauley LK, Somerman MJ, editors. Mineralized Tissues in Oral and Craniofacial Science. Hoboken, NJ: John Wiley & Sons, Inc.; 2010.

2. Rodrigues TL, Foster BL, Silvério KG, Martins L, Casati MZ, Sallum EA, Somerman MJ, Nociti, Jr. FH. Phosphate-regulating factors are differentially required for dental tissues homeostasis. (submetido no Journal of Dental Research)

3. Phosphate metabolism is a critical factor for periodontal regeneration. An *in vivo* study in mice. (a ser submetido no Journal of Periodontology)

SUMÁRIO

INTRODUÇÃO

A completa regeneração das estruturas periodontais perdidas em conseqüência à doença periodontal é um dos principais objetivos da terapia periodontal. Na busca de tratamentos que possam reverter a destruição causada pela doença, incluindo regeneração de novo osso, novo cemento e novo ligamento periodontal (PDL), aumentou-se o interesse em entender os mecanismos celulares e moleculares envolvidos, bem como os fatores que regulam esses tecidos durante o desenvolvimento e regeneração (Saygin et al., 2000, Bartold et al., 2000). Considerando a hipótese de que a regeneração periodontal mimetiza os episódios do desenvolvimento embrionário, pesquisas recentes têm sido feitas em modelos animais modificados geneticamente (genes bloqueados ou mutantes), com o objetivo de investigar as células e os moduladores necessários para a formação dos tecidos periodontais, potenciais indutores da regeneração periodontal (Beertsen et al., 1999; D'Errico et al., 2000; Harmey et al., 2004; Nociti et al., 2002; Takano et al., 2003). Uma destas linhas de pesquisa explora o efeito do fosfato na formação do cemento, investigando tecidos e células de camundongos com mutações genéticas que afetam os níveis de fosfato e pirofosfato no ambiente periodontal, objetivando aplicar esses conhecimentos no desenvolvimento de terapias que promovam a regeneração dos tecidos periodontais e orais.

Para que a deposição mineral normal ocorra nos ossos e dentes, é necessário um ajuste preciso entre os níveis extracelulares dos íons fosfato inorgânico (P_i) e pirofosfato inorgânico (PP_i), o qual é um potente inibidor da formação de cristais de hidroxiapatita (HAP). Três moléculas já foram identificadas como reguladoras-chaves dos níveis extracelulares de P_i e PP_i: a fosfatase alcalina (TNAP), que hidrolisa PP_i em P_i; a glicoproteína-1 ("*ectonucleotidepyrophosphatase-phosphodiesterase1"*- NPP1), que dá origem ao PP_i através da adenosina trifosfato (ATP) extracelular; e a proteína de anquilose (ANK), que media o fluxo intracelular e extracelular do PP_i (Anexo 1).

A investigação dos tecidos periodontais em desenvolvimento de camundongos com baixos níveis de fosfatase alcalina (condição correspondente à hipofosfatasia em

humanos - HPP) esclareceu a importância da expressão local desta enzima no início da cementogênese (Beersten et al., 1999). Nesses animais, e também nos indivíduos com HPP (Chapple, 1993), há um defeito na formação de cemento acelular sobre a superfície radicular. Além disso, há um aumento no nível de PP_i extracelular (ePP_i) e uma significativa diminuição na mineralização óssea (Fedde et al., 1999).

A hipofosfatasia (HPP) é uma desordem hereditária caracterizada pela disfunção da fosfatase alcalina, que resulta na perda prematura de dentes decíduos e/ou permanentes, bem como em anormalidades na mineralização do osso esquelético (Whyte, 2002). A prevalência das formas severas da doença é estimada em 1/100.000, atingindo todas as raças. A incidência da forma moderada nunca foi estimada, mas espera-se ser muito maior que a forma severa, uma vez que se tem um número de pacientes que carregam a forma dominante maior que os que possuem a forma recessiva (Whyte, 2010). Os sintomas clínicos são bem variados, os quais vão desde recém-nascidos sem mineralização óssea a perda prematura dos dentes sem nenhum sintoma ósseo. Dependendo da idade diagnosticada, seis formas clínicas são atualmente relatadas: perinatal (letal), HPP de início no pré-natal, HPP infantil, HPP durante a infância, HPP do adulto e odonto-hipofosfatasia. Na foram letal, os pacientes apresentam uma mineralização altamente deficiente no útero. Na forma com início no pré-natal apresentam os mesmos sintomas que a forma anterior, porém com melhoras espontâneas com o passar do tempo. Os sintomas clínicos da forma infantil são complicações respiratórias, craniossinostose prematura, desmineralização generalizada e sinais de raquitismo em regiões de metáfise. A HPP durante a infância é caracterizada por deformidades esqueléticas, pequena estatura, e modo de andar "cambaleando". A forma adulta é caracterizada pelas fraturas por estresse, dores nas pernas, e osteoartropatias. A odonto-HPP é caracterizada pela perda prematura dos dentes decíduos e por cáries severas, na maioria das vezes não associada com anormalidades do sistema esquelético. O prognóstico dessas seis formas da doença é determinado pelas complicações esqueléticas, ou seja, quanto mais precoce os sinais e sintomas, pior os resultados (Whyte, 2010). Considerando a relação existente entre os tecidos periodontais e a hipofosfatasia, estas informações podem, ainda, ser úteis no manejo dos pacientes portadores desta condição.

O efeito inibitório da deficiência de fosfatase alcalina na formação do cemento na condição de HPP motivou investigações em camundongos geneticamente modificados, apresentando mutações nos genes *ank* e *Enpp1* (Nociti et al., 2002). Foi demonstrado um grande aumento na formação do cemento nesses camundongos, que apresentavam baixos níveis de ePP_i como resultado da mutação genética. Outros estudos, ainda, demonstraram que o tratamento com 1-hidroxietilideno-1, 1-bisfosfonato (HEBP), um análogo do PP_i, diminuiu significativamente a mineralização de tecidos dentais, especificamente o cemento (Alatli-kut *et al.*, 1994; Takano *et al.*, 2003). Em contraste aos achados nos tecidos periodontais, os outros tecidos dentais, mais especificamente a dentina, não apresentam alterações significativas seja pela situação de falta ou excesso de P_i/PP_i no ambiente extracelular (Beertsen et al., 1999; Liu et al., 2010; Olsson et al., 1996; van den Bos et al., 2005). Desta forma, estes achados sugerem que as células do ambiente periodontal são altamente sensíveis e responsivas às alterações metabólicas do fosfato e pirofosfato em relação aos outros tecidos dentais. A compreensão dos mecanismos biológicos envolvidos torna-se, então, um passo importante para um melhor entendimento dos processos de formação e regeneração dos tecidos periodontais.

Diante do exposto, estes estudos tiveram como objetivos: i) apresentar dois casos clínicos de perda prematura de dentes decíduos diagnosticados como odonto-hipofosfatasia (odonto-HPP); ii) caracterizar diferencialmente o perfil de expressão gênica de células da polpa e ligamento periodontal em indivíduos saudáveis e com hipofosfatasia em relação a genes envolvidos com o metabolismo do P_i; iii) caracterizar o reparo e regeneração dos tecidos periodontais em modelo de fenestração periodontal realizado em camundongos apresentando o bloqueio do gene *Ank* (*Ank* konck-out).

CAPÍTULO 1

Cementum and periodontal defects resulting from odontohypophosphatasia predispose for premature tooth loss

Short title: Odontohypophosphatasia and cementum

One sentence summary: Developmental defects in cementum and other dental tissues resulting from odontohypophosphatasia can jeopardize primary and secondary dentition, but with early diagnosis and careful management, permanent teeth can be maintained.

ABSTRACT

Background: Hypophosphatasia (HPP) is a rare inherited disorder caused by mutations in the gene for the tissue nonspecific form of alkaline phosphatase (Alpl). HPP is variable in onset and severity, and is characterized by a continuum of mineralization defects in bones and teeth, including defective cementum development and early loss of primary and permanent teeth.

Case Presentation: Twin boys at two years of age suffered premature exfoliation of anterior teeth. Both exhibited low levels of serum alkaline phosphatase activity, but no additional skeletal abnormalities, prompting a diagnosis of odontohypophosphatasia (odonto-HPP). Pediatric dental care and supportive periodontal therapy were performed during the subsequent 19 years, aimed at avoiding or delaying loss of permanent teeth.

Conclusion: Premature loss of deciduous teeth in absence of skeletal disorders may serve as a critical trigger sign for diagnosis of odonto-HPP or other subtypes. The pediatric dentist is uniquely positioned to guide early diagnosis and initiate conservative care of affected patients. Odonto-HPP presents a complex challenge for clinicians, though careful management can successfully maintain permanent teeth.

INTRODUCTION

Hypophosphatasia (HPP) is an inherited disorder characterized by defective bone and tooth mineralization, and deficiency of serum alkaline phosphatase activity (ALP). The disease is a consequence of mutations in the liver/bone/kidney alkaline phosphatase gene (Alpl; OMIM 171760), encoding tissue-nonspecific alkaline phosphatase (TNAP). Biochemical manifestations include increased urinary phosphoethanolamine (PEA), increased plasma pyridoxal 5'-phosphate (PLP), and increased urinary pyrophosphate (PP_i). While PEA, PLP, and PP_i may be physiological substrates for TNAP, accumulation of the mineralization inhibitor PP_i has been identified as the proximal cause for mineralization defects (Whyte, 1994).

HPP is highly variable in onset and severity, ranging from stillbirths lacking mineralized bone, to milder forms marked by late onset bone complications, or tooth defects without bone complications. HPP has been associated with more than 200 different mutations in Alpl, and mode of inheritance can be autosomal dominant or recessive, though recessive patterns are more often identified and are associated with more severe symptoms. Prevalence of severe forms has been estimated at 1/100,000, while the incidence of mild to moderate forms is probably higher and more likely undiagnosed. HPP is currently classified into six clinical subtypes: perinatal (lethal), infantile, childhood, adult, and odonto-HPP, as well as a more rare benign prenatal form (Table 1) (Mornet, 2007; Mornet et al., 2010).

Most HPP subtypes feature dental abnormalities of varying severity (Reibel et al., 2009). Odonto-HPP is distinguished by premature exfoliation of fully rooted primary teeth in the absence of other skeletal manifestations. The anterior deciduous teeth are more likely to be affected, with incisors lost earliest and most frequently. The primary lesion involves a qualitative and quantitative defect of tooth root acellular cementum formation, though root resorption has also been reported (Bruckner et al., 1962; Chapple, 1993; van den Bos et al., 2005). Oral findings may represent the primary clinical manifestations in mild HPP cases (including childhood, adult, and odonto-HPP), and therefore premature exfoliation of primary teeth should be considered a *trigger sign* that may lead to a clinical diagnosis (Reibel et al., 2009).

CASE PRESENTATION

Male identical twins of Caucasian descent, aged two years old, were brought for dental evaluation by their parents to the University of Campinas, Dental School at Piracicaba, Brazil. Patients' parents reported premature exfoliation of the anterior primary teeth, with signs of partial root resorption. Examination indicated developmentally normal children, with no additional aberrant physical findings. Biochemical analysis revealed low serum ALP activity for both (patient A: 62 U/L, patient B: 63 U/L; normal range for children 151-471 U/L), while serum phosphate and calcium levels were within the normal range. Renal function, as evidenced by blood urea, creatinine, and urinalysis, was unaffected for both patients. Radiographs of the hands, wrists, and legs revealed age-appropriate growth and development, with well-preserved joint spaces. Based on available clinical, radiographical, and serum biochemistry, a diagnosis of odonto-HPP was given.

Mutation screening was performed by sequencing the 12 exons of the Alpl gene. A heterozygous transition 454C>T in exon 5 was identified in both patients, resulting in the substitution of arginine by cysteine at position 135 (R135C) (Weiss et al., 1988). While this change may be related to HPP, no correlation with ALP activity, clinical observations, or family historical data has yet been confirmed. This particular alteration has not been previously reported for odonto-HPP, though codon 135 has been linked to a case of adult-onset HPP (R135C/R167W, SESEP Laboratory and the Human Molecular Genetics laboratory of the University of Versailles-Saint Quentin en Yvelines, France), as well as to a lethal HPP case (R135H) (Taillandier et al., 2001), suggesting genotype-phenotype correlation. Alpl mutations and deletions associated with the odonto-HPP subtype have been localized to exons 4, 5, 6, 7, 8, 9, 10, 11, and 12, indicating no specific locus associated with this subtype.

The dental history indicated that patient A lost the lower left primary central incisor (tooth 71 by FDI notation) and patient B, the upper left primary central incisor (tooth 61) at approximately one year old. Losses were initially attributed to trauma because patients fell and injured their mouths. About six months following the first incident, remaining teeth were moderately mobile, and both patients experienced additional

spontaneous tooth exfoliation at intervals of about six months apart. Oral examination did not reveal any gingivitis or bleeding on probing, and remaining teeth included: 55, 54, 64, 65, 75, 73, and 85 for patient A, and 55, 53, 63, 65, 75, 73, 83, 84, and 85 for patient B. For both patients, multiple teeth featured enamel hypoplasia. Radiographs revealed premature root resorption in remaining deciduous teeth, but apparently normal development of the permanent dentition.

Partial dentures were inserted at three years old, immediately after loss of primary teeth because of the rapid mesial migration of adjacent teeth. From age three and up, recall visits were scheduled four times per year to monitor general oral health, hygiene, and update the treatment plan for any specific complications. All primary teeth were lost by age 7 (normal range 6-12 years), and eruption of some permanent teeth was delayed. At 14 years old, patient A experienced loss of the permanent mandibular central incisor (tooth 31) during brushing (Figure 1, A and B). The associated radiograph (Figure 1B) exhibited delayed eruption of several permanent teeth, which normally erupt at 10-12 years. Scanning electron microscopy on this tooth revealed cementum aplasia or severe hypoplasia, with large portions of root dentin exposed (Figure 1C). At 19 years old, the same patient suffered an athletic trauma fracturing central incisor, tooth 41 (Figure 2). The lower teeth were splinted with a provisory tooth replacing 41, and lateral incisors 42 and 32 were treated endodontically because of pulp necrosis. Radiographs demonstrate reduced alveolar bone as well as short roots and wide pulp chambers.

At age 15, patient B suffered an athletic trauma resulting in loss of tooth 41 and pulp necrosis in maxillary lateral incisor 22. At that time, the oral examination of both patients revealed relatively good hygienic condition, with only small amounts of supragingival calculus at gingival margins. In the following years, patients were kept on supportive periodontal therapy with recalls every three months. During visits, periodontal examinations included assessments of oral hygiene, gingival inflammation, probing depth (PD), clinical attachment loss (CAL), radiographs, and standard care, including oral hygiene instructions and mechanical nonsurgical debridement.

At age 20, a more critical periodontal condition was found. Bleeding on probing (BOP) was observed in 32% and 13% of the assessed sites (bucco-mesial, bucco-central,

bucco-distal, and lingo-central), 8.6% and 3.8% of sites presented CAL of 3-4 mm, and 9.6% and 4.8% sites presented CAL ≥5 mm, for patients A and B, respectively. Radiographically, reduced alveolar bone height was apparent in the regions of the incisors, while in the posterior region, bone loss was more significant in the area of the first molars, with the mandibular molars extensively involved (Figure 3). Patients were diagnosed with periodontitis as a manifestation of systemic disease, associated with a genetic disorder, and with localized extension and severe attachment loss (Armitage, 1999). Initial phase therapy, consisting of scaling and root planning, oral hygiene instructions, and patient education and motivation, was performed with recall every two months.

At present, after two years of this therapy regime, periodontal status remains stable with full mouth bleeding score and full mouth plaque score less than 15%. With a more stable periodontal condition, orthodontic treatment was initiated in both patients in order to provide more harmonic occlusion after tooth loss, and in preparation for dental implants. The ongoing orthodontic treatment employs minimal forces, with visits scheduled on a monthly basis, with some improvement already noted in occlusion (Figure 4). At completion of orthodontic treatment, implant-supported prostheses are planned to replace lost teeth. Biochemical follow-up exams showed that serum ALP activity remains low for both patients, at 8U/L and 6U/L, for patient A and B, respectively (normal range for adult 25-100 U/L).

DISCUSSION

Phosphate (P_i) homeostasis is critical to the normal development, maintenance, repair, and regeneration of mineralized tissues, including teeth (Foster et al., 2008). In the context of mineralization, P_i metabolism is tempered by the actions of pyrophosphate (PP_i), a potent inhibitor of hydroxyapatite crystal precipitation. Studies in recent years indicate that the diverse mineralized tissues of the skeleton and teeth (i.e. bone, cementum, dentin, and enamel) are subject to differential regulation by prevailing P_i/PP_i conditions (Foster et al., 2008; van den Bos et al., 2005). Cementum of the tooth root is especially sensitive to dysregulation of local PP_i homeostasis, based on dramatic phenotypes observed in

transgenic mice wherein PP_i levels are increased or decreased (Beertsen et al., 1999; Foster et al., 2008; Nociti et al., 2002)

Reduced TNAP function in hypophosphatasia (HPP) results in defective bone and tooth mineralization, with severity of clinical presentation associated with dominant or recessive inheritance, age of onset, and extent of reduction of ALP activity (Mornet, 2007; Whyte, 1994). HPP provided the first clue linking PP_i metabolism disorder with a developmental cementum phenotype. TNAP deficiency causes aplasia or severe hypoplasia of the acellular cementum (acellular extrinsic fiber cementum, AEFC) (Bruckner et al., 1962; Chapple, 1993; van den Bos et al., 2005). Acellular cementum is critical for tooth attachment by anchoring the root to the surrounding alveolar bone via the periodontal ligament (PDL). When AEFC is deficient, as in HPP, Sharpey's fibers from the PDL are poorly developed and insecurely embedded, resulting in increased tooth mobility and susceptibility to exfoliation. The rich expression of TNAP in periodontal tissues, as well as developmental analysis of Akp2 (mouse homologue of Alpl) knock-out mice, has further highlighted the importance of local expression of this enzyme for initiation of cementogenesis (Beertsen et al., 1999; Groeneveld et al., 1995). HPP affects other tissues of the dentoalveolar complex (Table 1). Alveolar bone mineralization may be disrupted by loss of ALP activity resulting in hyperosteoidosis, as well as being secondarily affected by exfoliation of teeth and loss of occlusal loading. Some case reports have described dentin defects including short roots, thin dentin, and abnormally wide pulp chambers, as well as enamel hypoplasia and susceptibility to caries (Hu et al., 2000; Reibel et al., 2009). No effective treatment currently exists for HPP, but TNAP enzyme replacement therapy looks promising for both skeletal and dental defects (Millán et al., 2008).

In the two cases reported here, premature loss of primary dentition served as a trigger sign for diagnosis of HPP. Decreased serum ALP activity and lack of additional mineralization disorders narrowed the focus to the odonto-HPP subtype. Patients presented additional features suggestive of odonto-HPP, including reduced alveolar bone height, enlargement of coronal and root pulp chambers, enamel hypoplasia in primary teeth, and delayed eruption of permanent teeth. SEM imaging of one of the exfoliated permanent teeth confirmed cement defects and extensive root resorption.

Odontohypophosphatasia presents a complex challenge for clinicians. The pediatric dentist faced with early signs of odonto-HPP is uniquely positioned to guide early diagnosis and initiate conservative care of affected patients. However, the difficult question arises of what prognosis is expected, and correspondingly, what manner of care is recommended? The variable severity of HPP makes this is a difficult question to answer, though case reports highlighting long-term dental care of HPP patients can begin to provide some guidance on this issue. A fair long-term dental prognosis for a patient with infantile HPP and a severe deciduous dental phenotype has been reported (Reibel et al., 2009). Though this patient lost all deciduous teeth by 8 years old, and secondary teeth featured crown and root abnormalities, most of the permanent teeth were still present after 20 years follow-up, and alveolar bone height held stable. Pulp chambers featured secondary dentin apposition, suggesting delayed dentin formation or mineralization and the possibility for partial correction of developmental defects with time. In some cases, permanent teeth seem wholly unaffected, even with a severe phenotype in deciduous teeth (Lepe et al., 1997). Conversely, other clinical descriptions of HPP paint a less optimistic picture where both primary and secondary dentitions are severely affected, resulting in a much less favorable outcome (el-Labban et al., 1991; Macfarlane and Swart, 1989; Olsson et al., 1996).

As suggested by the HPP literature, it remains difficult to predict the prognosis for the permanent dentition based on developmental disturbances in primary teeth. Therefore, for the patients described here, a systematic follow-up program was initiated, including frequent visits, oral hygiene instructions, periodontal maintenance, and operative actions, when required. After initial premature losses of some permanent teeth, a more stable periodontal status was established, and orthodontic treatment initiated to improve occlusion and prepare for dental implant placement in the future.

CONCLUSIONS

Premature loss of deciduous teeth in absence of skeletal disorders may serve as a critical trigger sign for diagnosis of odonto-HPP or other subtypes. Thus, the pediatric dentist is uniquely positioned to guide early diagnosis and initiate systematic and

conservative care of affected patients, which can successfully maintain permanent teeth over a longer period of time. If a degree of periodontal health and stability can be attained, orthodontic treatment and implant-supported prostheses may also be considered in order to restore function and esthetic lost as a consequence of early exfoliation of permanent teeth.

ACKNOWLEDGEMENTS

We gratefully acknowledge support from the São Paulo State Research Foundation (FAPESP, São Paulo, SP, Brazil), grant numbers 07/08192-5 and 08/00534-7.

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TABLE:

Table 1: Clinical subtypes of hypophosphatasia.

Clinical subtype	Bone symptoms	Dental symptoms and features	Diagnostics
Perinatal lethal	Severe hypomineralization Osteochondral spurs on arms and legs Rachitic ribs and respiratory complications	N/A	Ultrasonography Radiographs
Prenatal benign	Bowing, shortening of long bones Improvement of symptoms in third trimester or postpartum	N/A	Ultrasonography Clinical examination
Infantile	Generalized hypomineralization Bowing, shortening of long bones Craniosynostosis Rachitic ribs and respiratory complications Hypercalciuria	Premature loss of deciduous teeth Crown and root developmental defects (see odonto-HPP description)	Clinical examination Radiographs Biochemistry
Childhood	Short stature Development of skeletal deformities Delayed walking, waddling gait Fractures and bone pain	Premature loss of deciduous teeth Crown and root developmental defects (see odonto-HPP description)	Clinical examination Radiographs Biochemistry
Adult	Stress fractures of the metatarsal and tibia Possibility of osteoarthritis or chondrocalcinosis	Possible premature loss of deciduous teeth Possible loss of permanent teeth Crown and root developmental defects (see odonto-HPP description)	Clinical examination Biochemistry
Odonto-HPP	Loss of alveolar bone (may be secondary to tooth defects)	Premature loss of deciduous and/or permanent teeth Delay in eruption Lack of acellular cementum Dentin/pulp defects: reduced dentin thickness, enlarged pulp chambers Enamel hypoplasia, crown abnormalities, dental caries	Clinical examination Radiographs Biochemistry

N/A: not applicable

FIGURES:

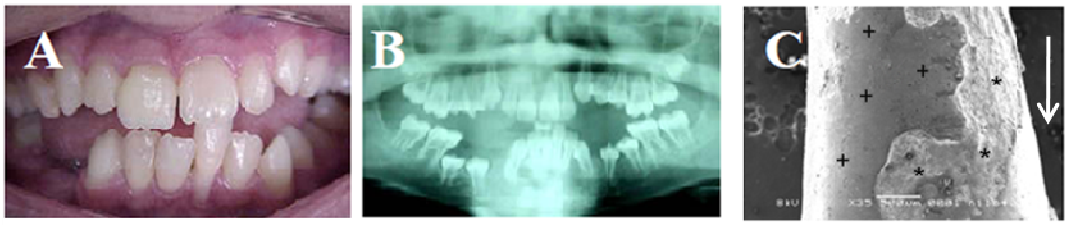

Figure 1: At 14 years old, patient A (a patient with HPP), experienced spontaneous exfoliation of the permanent mandibular central incisor (tooth 31 by FDI notation) during brushing**.** (A) Clinical photograph and (B) corresponding panoramic radiograph from patient A, showing effects of odonto-HPP on the permanent dentition, including delayed eruption of several permanent teeth (e.g. mandibular premolars 34, 35, 44, and 45), enlarged pulp chambers, and thin dentin. (C) SEM imaging of the apical root region of tooth 31 demonstrates lack of cementum and exposed dentin surface with no indication of attached collagenous PDL fibers (+), as well as evidence of root dentin resorption (stars). Arrow indicates the occlusal direction.

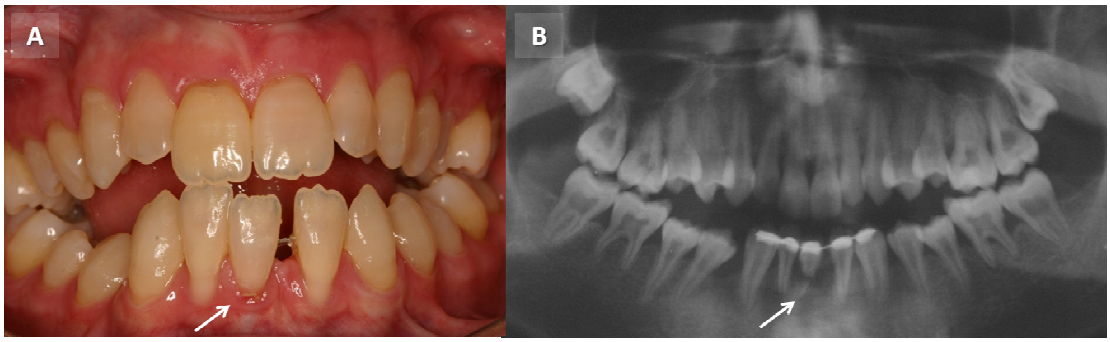

Figure 2: At 19 years of age, patient A suffered a fracture of mandibular central incisor 41 as a result of athletic trauma. (A) Clinical photograph showing malocclusion and open bite. (B) Corresponding panoramic radiograph displaying the fractured tooth (white arrow) and areas of reduced alveolar bone height, as well as short roots and wide pulp chambers.

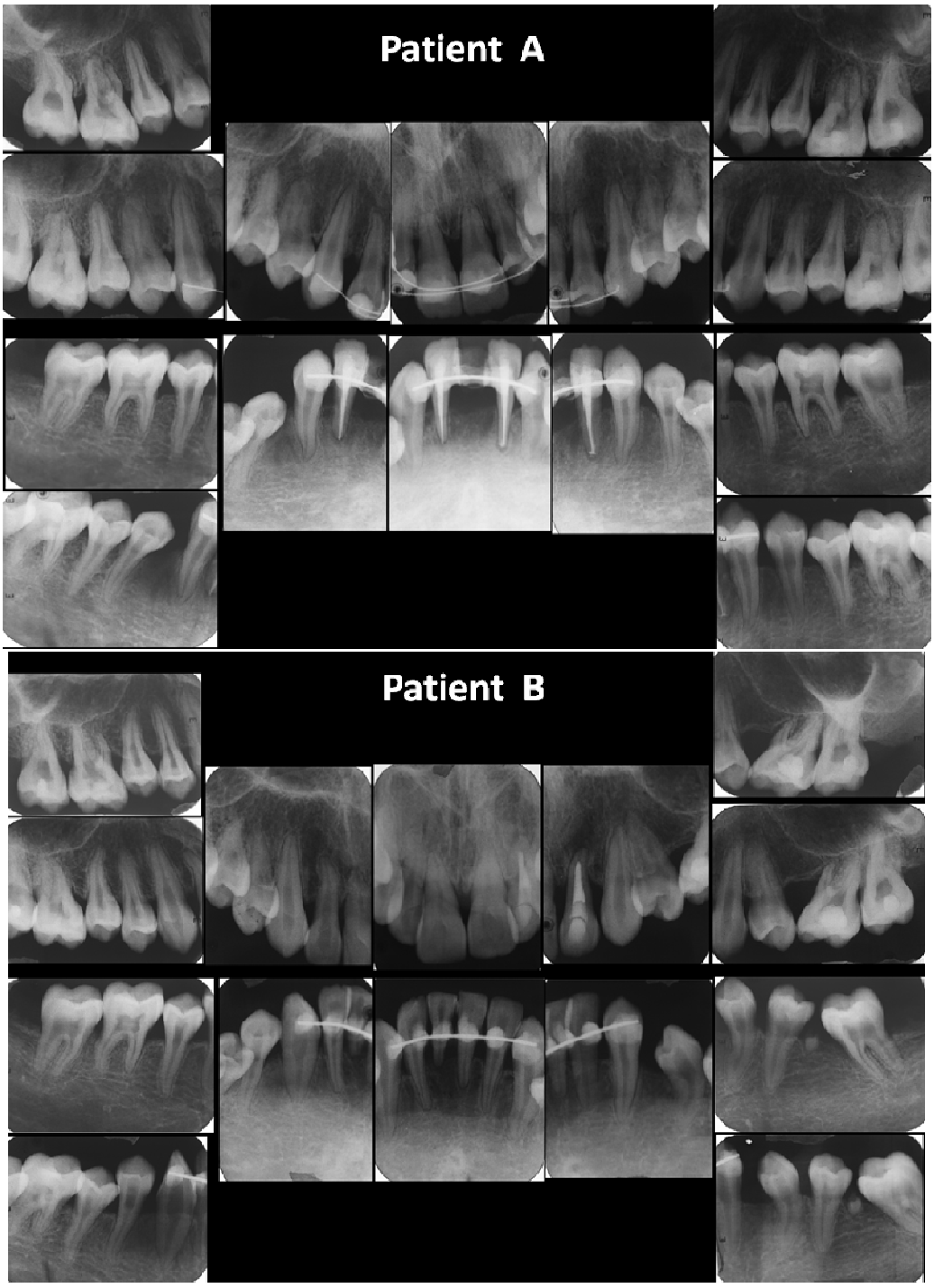

Figure 3: Full mouth periapical radiographs of patients A and B at 20 years of age, showing reduced alveolar bone height, short root length, and abnormally wide pulp chambers and root canal systems in several teeth.

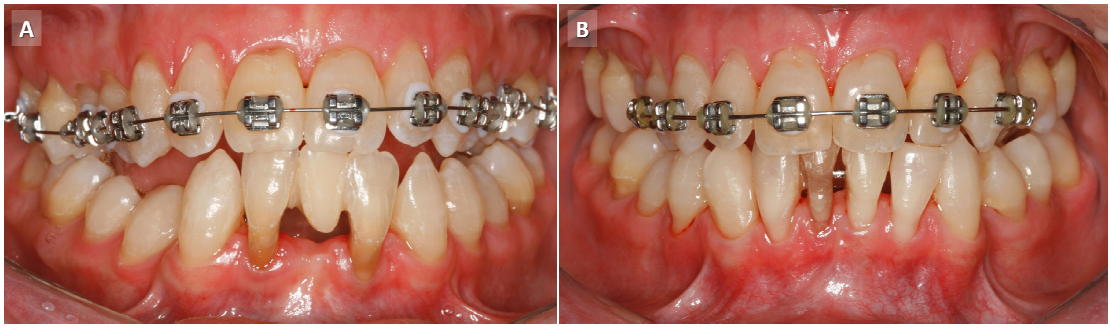

Figure 4: Clinical photographs of patient A and B during the active phase of orthodontic treatment to improve occlusion and prepare for dental implants. Orthodontic treatment is currently ongoing.

CAPÍTULO 2

Phosphate-regulating factors are differentially required for dental tissues homeostasis

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Words in abstract: 195
Total words: 2,495
Figures: 4
Tables: 1 Supplemental
References: 30
Running title: Periodontal Ligament and Pulp Cells in Hypophosphatasia
Key words: dental pulp, periodontal ligament, phosphate metabolism, hypophosphatasia

Funding: São Paulo State Research Foundation (FAPESP, Brazil, grants #07/08192-5 and #08/00534-7), and NIH FIRCA (grant # 5R03TW007590-03)

ABSTRACT

Biomineralization requires a tight balance between levels of inorganic phosphate (P_i) and pyrophosphate (PP_i). P_i/PP_i dysregulation during tooth development results in tissue-specific defects. In hypophosphatasia (HPP), where tissue-nonspecific alkaline phosphatase (TNAP) function is reduced, acellular cementum formation is severely defective, while dentin is reported to be unaffected or variably affected. *Ex vivo* and *in vitro* analysis were employed to identify mechanisms involved in these divergent responses. Constitutive expression of P_i/PP_i-associated genes in periodontal ligament (PDL) versus pulp tissues obtained from healthy subjects were analyzed. Primary cell cultures from control and HPP-PDL and pulp tissues were established to assay mineralization and gene expression. In healthy tissues, PDL maintained significantly higher basal expression of key PP_i regulators, liver/bone/kidney alkaline phosphatase (Alpl), progressive ankylosis protein (Ank) and ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1), versus pulp. *In vitro*, although more dramatic changes were found for PDL-harvested cells, both HPP-PDL and HPP-pulp cells exhibited significantly lower alkaline phosphatase activity, mineralization, and depressed expression of genes associated with mineralization and regulation of P_i/PP_i, versus control cells. These findings suggest PDL cells are more severely affected by loss of TNAP than pulp cells and may explain the more dramatic PDL/root versus pulp phenotype in HPP individuals.

INTRODUCTION

Biomineralization requires a tight balance between extracellular phosphate (P_i) and pyrophosphate (PP_i) concentrations. Disorders related to unbalanced P_i/PP_i have been reported to produce dissimilar effects on developing dental hard tissues, and have prompted the hypothesis that dental tissues are differentially sensitive to P_i metabolism. In hypophosphatasia (HPP), deficiency of serum alkaline phosphatase activity (ALP) results from mutations in the liver/bone/kidney alkaline phosphatase gene (Alpl; OMIM 171760), encoding tissue nonspecific alkaline phosphatase (TNAP). One consequence is defective

formation of acellular cementum resulting in poor attachment and premature tooth exfoliation (Chapple, 1993). In the *Akp2* null murine model for HPP, cementum is similarly defective (Beertsen et al., 1999). Yet in both humans and mice with reduced ALP, dentin has been reported to be unaffected or minimally affected (Beertsen et al., 1999; Liu et al., 2010; Olsson et al., 1996; van den Bos et al., 2005). Accumulation of mineralization inhibitor PP_i has been identified as the proximal cause for defective mineralization in dental hard tissues. In contrast, in murine models where local PP_i is deficient, namely loss of progressive ankylosis (ANK) or ectonucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) function, acellular cementum is markedly increased, while dentin is unaffected (Foster et al., 2011; Nociti et al., 2002).

Although differences in P_i/PP_i regulators between periodontal and pulp tissues in healthy subjects have been suggested (van den Bos et al., 2005), the mechanism for differential effects of P_i/PP_i dysregulation on cementum and dentin remains unclear. Here, we hypothesized that P_i-regulating factors are differentially required for dental tissues homeostasis. We determined whether or not differences existed in expression of P_i/PP_i regulators in a healthy state between periodontal and pulp tissues, and expanded to mechanistic studies through analysis of *in vitro* cell culture systems of primary HPP-PDL and -pulp cells.

MATERIALS AND METHODS

Human subjects

A total of 16 patients (10 females) 17-22 years old were enrolled in this study, with Institutional Review Board approval. Inclusion criteria were no history of smoking, diabetes, bone metabolic disorders or other systemic disease, except for hypophosphatasia in the HPP group. For the control group, patients were periodontally healthy with teeth scheduled for extraction for orthodontic reasons, and with serum alkaline phosphatase activity (ALP) within the normal adult range (25-100 U/L). HPP subjects were monitored and treated as needed, and tooth extractions were performed as a consequence of HPP-related pathology.

Genetic analysis of HPP patients

DNA from blood samples was purified from leukocytes (Aidar and Line, 2007). Twelve exons of Alpl (liver/bone/kidney alkaline phosphatase, OMIM 171760) were PCR-amplified (Mornet et al., 1998). Sequencing analyses were performed with an ABI DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare UK Limited, Buckinghamshire, UK) on an ABI PRISM^{TM} 3100 sequencer (Applied Biosystems).

Tissue harvest

Teeth were extracted from control patients (n=9), rinsed in PBS, and PDL tissues removed by scraping the root surface. After cracking open the teeth, pulp tissue was harvested with sterile forceps. Tissues were immersed in RNAlater® (Ambion Inc., Austin, TX) and stored at -80°C. Total RNA was isolated by ceramic bead homogenization with TRIZOL® reagent (Gibco BRL) using the MagNA Lyser (Roche Applied Science, Indianapolis, IN).

Cell isolation and culture

Extracted teeth were placed in biopsy media, and patient-matched PDL and pulp cells (n=5 control, n=2 HPP patients) were obtained by enzymatic digestion (3mg/ml collagenase type I, 4 mg/ml dispase, Gibco BRL) for 1h at 37°C. Cells were maintained in DMEM with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin (Gibco BRL), incubated at 37°C in 5% CO_2. Equivalent passages 2-4 were used for experiments.

Cell proliferation

Cells were seeded at $1.5x10^4$ cells/cm^2 in 96-well plates in DMEM 2% FBS from 24h to 6 days. Cells were counted by hemacytometer and analyzed by an MTS-based cell proliferation assay (Promega, Madison, WI).

Alkaline phosphatase activity

Cells were seeded at 2.0×10^4 cells/cm^2 in 60 mm plates in DMEM 2% FBS and 50 µg/ml ascorbic acid (AA) to 21 days. Extracellular ALP activity was measured using a commercial kit (Labtest Diagnostica, MG, Brazil), as previously reported (Rodrigues et al., 2007).

Mineralization assay

Cells were seeded at 2.0×10^4 cells/cm^2 in 24-well plates under non-mineralizing (2% FBS) or mineralizing conditions [2% FBS, 50 μg/ml AA, and 10 mM β-glycerophosphate (βGP, Sigma, St. Louis, MO)] up to 28 days. Mineral nodules were detected by von Kossa (VK) assay and alizarin red-S staining (AR, 40mM, pH4.2; Sigma). AR stain was quantified by measuring absorbance of bound dye (570 nm) solubilized in 10% cetylpyridinium chloride (MP Biomedicals, Solon, OH) (Hessle et al., 2002).

Gene expression

Cells were seeded at 2.0×10^4 cells/cm^2 in 60 mm plates in DMEM 2% FBS and 50 µg/ml AA up to 15 days. Total RNA was extracted using Trizol® reagent (Gibco), DNase treated (Turbo DNA-free®, Ambion, Austin, TX), and used for cDNA synthesis (Roche Diagnostic, Indianapolis, IN, USA). Quantitative real-time PCR reactions were performed using SYBR Green (FastStart DNA Master^{plus}, Roche Diagnostic). Relative quantification was performed using amplification efficiency correction with glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as the reference gene. Assessed genes and primers are listed in Supplemental Table 1.

Statistical analysis

Experiments were performed in triplicate and repeated at least twice. Values are given as means and standard deviation (SD). Intragroup and intergroup comparisons were performed using the Kruskal-Wallis one-way analysis of variance followed by the Student-Newman-Keuls method (α=0.05) for proliferation and mineralization assays. Student t-test was used for intra and intergroup comparisons for ALP activity and intergroup comparisons

(control versus HPP) for gene expression within the same cell group (PDL or pulp) and experimental period. Statistical power was at least 0.80 with α=0.05 for any statistical test.

RESULTS

Diagnosis of hypophosphatasia

HPP individuals were evaluated for possible mineralized tissue disorders at the age of 2 because of premature exfoliation of anterior primary teeth. Physical examination and radiography (long bones, joints, and skull) did not indicate additional aberrant findings. Biochemical analysis revealed low serum ALP activity (patient A: 62 U/L, patient B: 63 U/L; normal range for children 151-471 U/L). Serum phosphate and calcium serum levels were normal. Based on the above, a diagnosis of odontohypophosphatasia was given.

Over subsequent years, patients suffered premature exfoliation of primary and permanent teeth. Permanent dentition featured short roots, wide pulp chambers, and reduced alveolar bone height (Figure 1A-C). At age 22, when cells were harvested for this study, ALP activity remained low (8U/L and 6U/L, for patient A and B, respectively; normal range 25-100 U/L).

Sequencing Alpl revealed a heterozygous transition 454C>T in exon 5 of both patients, leading to substitution of cysteine for arginine at position 135 (R135C) (Figure 1D). The missense mutation was confirmed by *HhaI* digest, demonstrating the expected restriction site was abolished (Figure 1E).

P_i/PP_i regulators are differentially expressed in PDL versus pulp tissues

Dissimilar effects of mineralization disorders on individual dental hard tissues during formation have prompted the hypothesis that cementogenesis and dentinogenesis are regulated by disparate mechanisms (Nociti et al., 2002; van den Bos et al., 2005). Constitutive gene expression was compared in PDL versus pulp tissues harvested from healthy subjects. PDL tissues featured significantly higher mRNA levels for Alpl, Ankh, and Enpp1, all key regulators of local PP_i levels ($p<0.05$) (Figure 2). Analysis of additional mineralized tissue markers revealed that PDL featured 10-fold higher expression of Ocn,

while Dmp1 was expressed at twice the levels in pulp versus PDL. Col1 was almost 20-fold higher in PDL versus pulp. No differences in Dspp and Mepe expression were found ($p>0.05$).

Dental cells from HPP patients exhibit reduced ALP activity and mineralization capacity

In light of basal differences observed in PDL versus pulp tissues, primary cell cultures were established to define dissimilarities between cells from healthy versus HPP individuals. PDL and pulp cells from controls and HPP patients showed a typical spindle-shaped fibroblastic morphology and monolayer attachment. Additionally, direct cell counting and MTS assay indicated proliferation and cell viability were comparable in control versus HPP cells (Supplemental figure 1).

ALP activity in HPP-PDL and pulp cells was decreased versus respective controls ($p<0.05$), but comparable to each other (Figure 3A). PDL and pulp control cells, under mineralization conditions, produced mineral nodules by 28 days, with PDL cells exhibiting greater mineralization (Figure 3B). HPP cells displayed a severely limited mineralization capacity. AR staining revealed 6- and 3-fold greater mineral formation in control versus HPP cells harvested from PDL and pulp, respectively ($p<0.05$) (Figure 3C).

HPP-PDL and pulp cell phenotypes are altered

To provide insight into capacity of HPP cells to regulate P_i/PP_i, differentiate, and mineralize, expression levels for associated genes were tracked in culture (Figure 4). Both HPP-PDL and pulp cells displayed parallel changes in several genes, including significantly decreased Ankh, Bsp, Dmp1, Dspp, and Mepe ($p<0.05$). Interestingly, HPP-PDL cells featured a significant increased Alpl expression, likely feedback attempting to compensate for ALP deficiency. Enpp1 transcripts were higher in HPP-pulp cells versus control, perhaps related to the decreased levels of Ankh. While HPP-PDL expressed Opn levels comparable to controls, except for day 15, HPP-pulp cells exhibited severely depressed Opn throughout ($p<0.05$).

DISCUSSION

Dissimilar effects of mineralization disorders on formation of cementum versus dentin suggest these tissues are subject to different developmental influences. It has been suggested that periodontal tissues fall more under the influence of PP_i metabolism than pulp/dentin (Nociti et al., 2002; van den Bos et al., 2005). Here we confirm that in health, periodontal tissues express higher basal levels of PP_i-regulating genes than pulp, as measured in *ex vivo* tissues. Differences in P_i/PP_i metabolism in dental cell populations were further defined employing PDL and pulp cultures from HPP patients. HPP-PDL and pulp cells exhibited low ALP activity and reduced ability to promote mineralization. Both HPP-PDL and pulp cells exhibited depression of differentiation/mineralization markers. The particular R135C alteration described here has not been previously reported for odonto-HPP, though codon 135 was linked to a case of adult-onset HPP (R135C/R167W; SESEP - University of Versailles-Saint Quentin), as well as to a lethal HPP case (R135H) (Taillandier et al., 2001), suggesting genotype-phenotype correlation.

Phosphate/pyrophosphate regulation in dental tissues

P_i homeostasis is essential for development and maintenance skeletal tissues, including the dentition (Foster et al., 2008). In mineralized tissues, P_i is compounded with calcium as hydroxyapatite (HAP). Conversely, PP_i is a potent inhibitor of HAP crystal growth (Fleisch and Bisaz, 1962). Local regulation of P_i and PP_i levels thus dictate mineralization, and hydrolysis of PP_i liberates P_i to relieve mineralization inibition. The enzyme TNAP hydrolyzes PP_i to P_i. In HPP, TNAP function is reduced, causing skeletal mineralization defects including rickets and osteomalacia (Mornet, 2007; Whyte, 1994). Dental case reports indicate compromised periodontal attachment and exfoliation of teeth due to aplasia or severe hypoplasia of the acellular cementum (Bruckner et al., 1962; Chapple, 1993; Hu et al., 2000; van den Bos et al., 2005). Dentin has been described as normal, or variably affected in HPP, with reports of thin dentin, wide pulp cavities, or "shell teeth" (Beumer et al., 1973; Liu et al., 2010; Olsson et al., 1996; Wei et al., 2010). In studies on *Akp2* null mice, a model for HPP, acellular cementum was inhibited while dentin

was unaffected (Beertsen et al., 1999). The progressive ankylosis protein (ANK) and ectonucleotide pyrophosphatase phosphodiesterase 1 (NPP1) both increase extracellular PP_i (Harmey et al., 2004; Hessle et al., 2002; Ho et al., 2000). In both *Ank* and *Enpp1* null mice, cementum was markedly increased, while dentin was unaltered (Foster et al., 2011; Nociti et al., 2002).

Studies to identify the mechanism for disparity in mineral metabolism of cementum versus dentin yielded some clues. van den Bos *et al.* reported higher Enpp1 gene expression, NPP-like activity, ALP activity, and PP_i concentrations in PDL versus pulp (van den Bos et al., 2005). Following on this work, we have identified higher expression of the primary PP_i regulatory factors Tnap, Ankh, and Enpp1 in PDL versus pulp in our normal sample population. These data suggest that PP_i metabolism in the PDL is a dynamic process, possibly predisposing periodontia to be highly sensitive to changes in PP_i regulators. This may be tied to the high rate of turnover of PDL, reflected here by about 20-fold greater expression of Col1 in PDL versus pulp.

Loss of TNAP function affects PDL and pulp cell phenotypes

To elucidate the mechanism for differential P_i/PP_i effects on cementum versus dentin, PDL and pulp cultures were obtained from healthy and HPP individuals. One limitation of this approach was our reliance on two HPP patients for cell isolation. As a rare disease, we were not able to identify additional individuals. The patients described here were identified with an odonto-specific subtype of HPP, where serum biochemistry is abnormal and dental defects are the primary phenotypic traits (Mornet, 2007; Whyte, 1994).

No differences were noted in morphology, proliferation, or viability between HPP versus control cells, in agreement with studies of HPP dermal fibroblasts (Fedde and Whyte, 1990; Whyte et al., 1983; Whyte and Vrabel, 1987). ALP activity and mineralization capacity for both HPP-PDL and pulp cells were significantly impaired, as reported for osteoblasts from *Akp2* null mice (Wennberg et al., 2000).

To our knowledge, this is the first report of *in vitro* gene expression analysis of PDL and pulp cells from HPP individuals. Because our hypothesis was that HPP-PDL cells

would exhibit greater alterations resulting from PP_i dysregulation, we elected to assay genes involved in P_i/PP_i regulation (Alpl, Ankh, Enpp1) and in cementoblast/osteoblast/odontoblast differentiation/function (Bsp, Dmp1, Dspp, Mepe, Opn). Both HPP-PDL and pulp cells featured reduced expression of Ankh and most differentiation/mineralization genes assayed, suggesting cell phenotype deficits resulting from the Alpl mutation. One notable exception was Opn, where HPP-PDL cells expressed similar or higher levels than controls, while HPP-pulp expressed significantly lower Opn across all times points. As OPN is an extracellular matrix protein shown to be an inhibitor of mineralization (Boskey et al., 1993), this divergence may point to relevant differences in responses of periodontal and pulp cell populations to TNAP dysfunction. Studies in *Akp2* null mice found that increased *Opn* mRNA in calvarial osteoblasts and greater OPN serum levels were in part responsible for bone hypomineralization (Harmey et al., 2004; Harmey et al., 2006). The response of tooth cells to PP_i dysregulation from HPP-pulp contrasts what has been reported in osteoblasts, highlighting local differences in the dental tissues.

Intriguingly, deficiencies in ALP activity, mineralization ability, and gene expression in HPP-PDL and pulp cells largely paralleled one another, deviating from our hypothesis that PDL cells would exhibit greater disruption. While the disrupted phenotype in HPP-pulp cells suggests a mechanism for HPP dentin phenotypes, data do not elucidate differences in severity of cementum vs. dentin defects. Studies are currently underway to determine if phenotypic changes in HPP-PDL and pulp cells result from inherent deficiencies, or if exogenous correction of P_i/PP_i metabolism will correct gene expression and mineralization in these cells. Both P_i and PP_i produce signaling effects on cells, as well as playing antagonistic roles in biomineralization (Addison et al., 2007; Foster et al., 2006). Insights into such roles of P_i/PP_i on dental development and function not only identify pathological mechanism, but may inform regenerative therapies.

ACKNOWLEDGMENTS

Studies were supported by São Paulo State Research Foundation (FAPESP 07/08192-5 and 08/00534-7) and NIH FIRCA (5R03TW007590-03). We thank Mrs. Ana Paula Giorgetti for clinical support and Dr. Marisi Aidar for genetic analyses.

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FIGURES

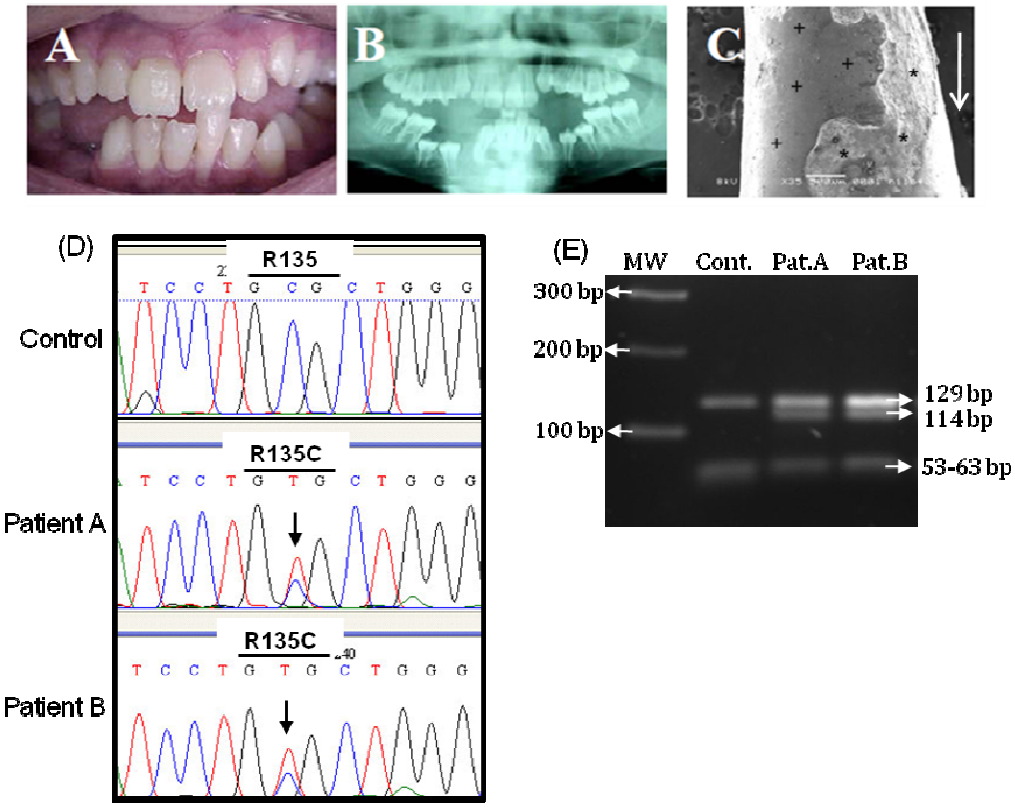


Figure 1. Hypophosphatasia (HPP) patients. (A) At 14 years old, patient A experienced spontaneous exfoliation of a permanent mandibular central incisor (tooth 31 by FDI notation) during brushing. **(B)** Corresponding panoramic radiograph, showing effects of odonto-HPP on the permanent dentition, including delayed eruption of several permanent teeth (e.g. mandibular premolars 34, 35, 44, and 45), enlarged pulp chambers, and thin dentin. **(C)** SEM imaging of the apical root region of tooth 31 demonstrates lack of cementum and exposed dentin surface with no indication of attached collagenous PDL fibers (+), as well as evidence of root dentin resorption (stars). Arrow indicates the occlusal direction. **(D)** Representative DNA sequencing electropherogram showing sequencing analysis of exon 5 in control versus patients A and B. The control subject is homozygous for C in the second position of codon 135 (454 nt), while the HPP patients are heterozygous, presenting both a normal (C) and altered allele (T) in the 454-nt position (R135C). Arrow indicates the position of nucleotide substitution in codon 135 GCG (Arginine) > GTG (Cysteine). **(E)** Confirmation of missense mutation was performed by enzymatic digestion, demonstrating the *Hha* I restriction site was abolished. MW: molecular weight marker.

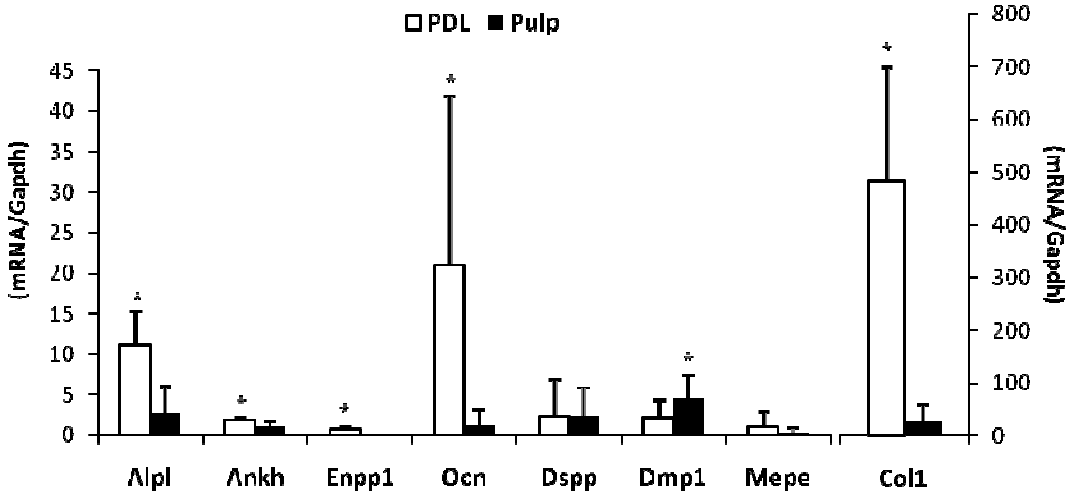


Figure 2. Expression of P_i/PP_i and mineralization related genes in PDL and pulp tissues. Gene expression in PDL and pulp tissues from 9 normal subjects (17-22 years old, 2 males and 7 females). Mean and SD for the mRNA levels of Alpl, Ankh, Enpp1, Ocn, Dspp, Dmp1, Mepe and Col1. The values expressed here are from two independent experiments, each conducted in triplicate. (*): statistically different by the Student t-test (alpha=0.05).

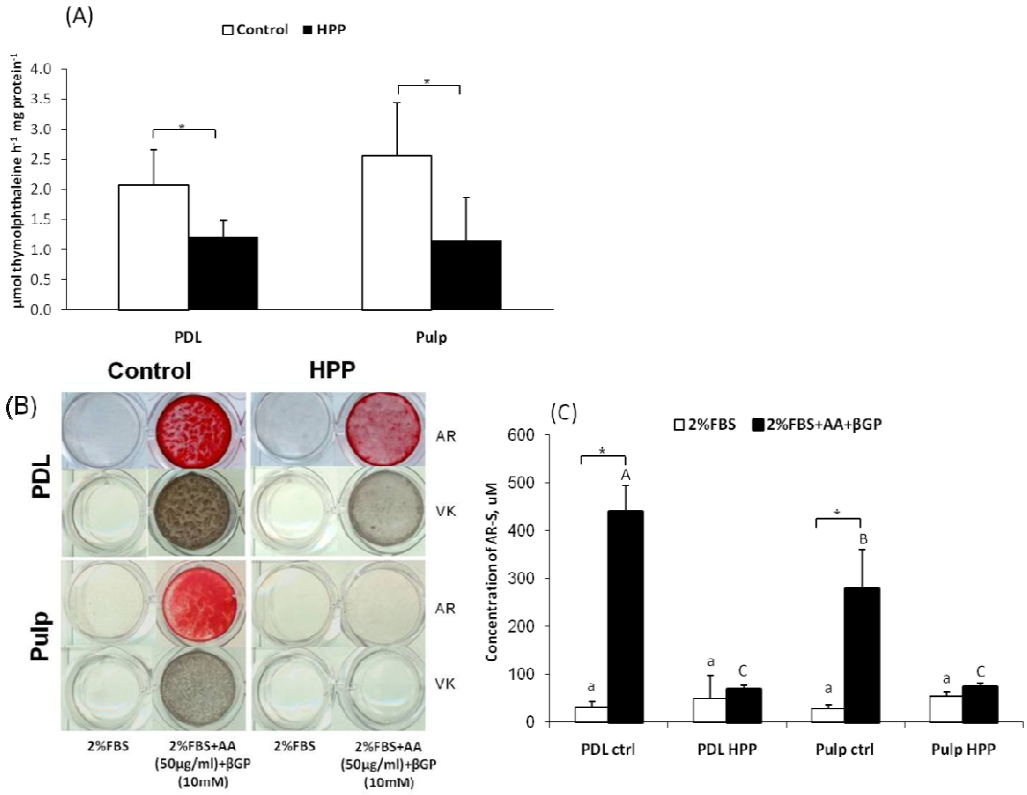


Figure 3. Alkaline phosphatase activity (ALP) and *in vitro* mineralization by control and HPP-PDL and pulp cells. (A) ALP activity in PDL and pulp control cells was higher when compared with HPP cells. (*): statistically different by the Student t-test (alpha=0.05). **(B)** Capacity of control and HPP PDL and pulp cells to promote mineralization *in vitro*. The photo of alizarin red (AR) and von Kossa (VK) staining for mineral nodules indicates that control cells produce nodules under mineralization conditions, with PDL exhibiting more capacity for mineralization than pulp, while HPP cells lose ability to mineralize under these conditions. **(C)** Quantification of AR stain with values expressed as mean ± SD (5 control and 2 HPP patients) in two independent experiments, each conducted in triplicate. Capital and non-capital letters indicate statistical differences using the one-way ANOVA followed by the Student-Newman-Keuls method (alpha=0.05) for mineralizing and non-mineralizing conditions, respectively.

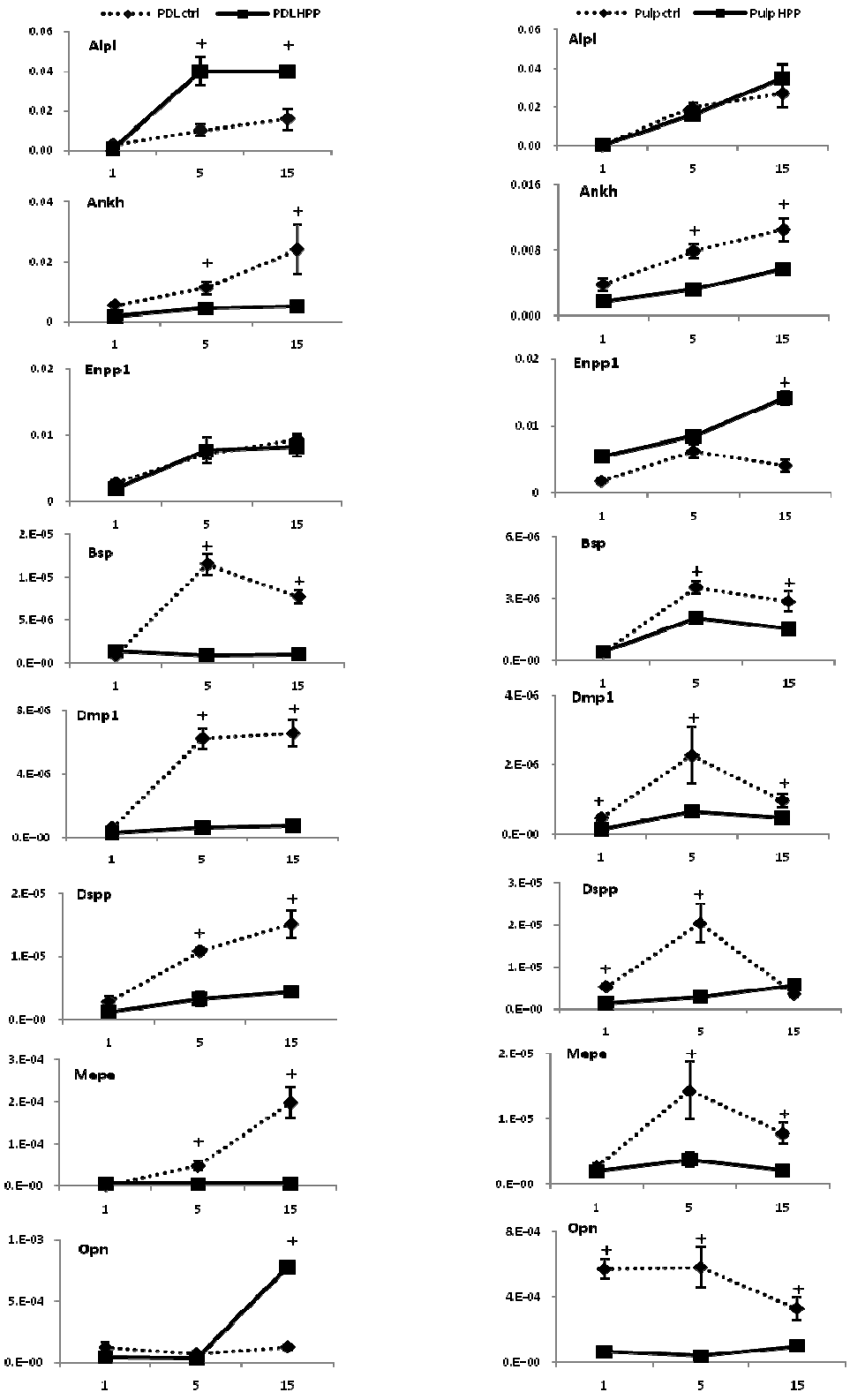
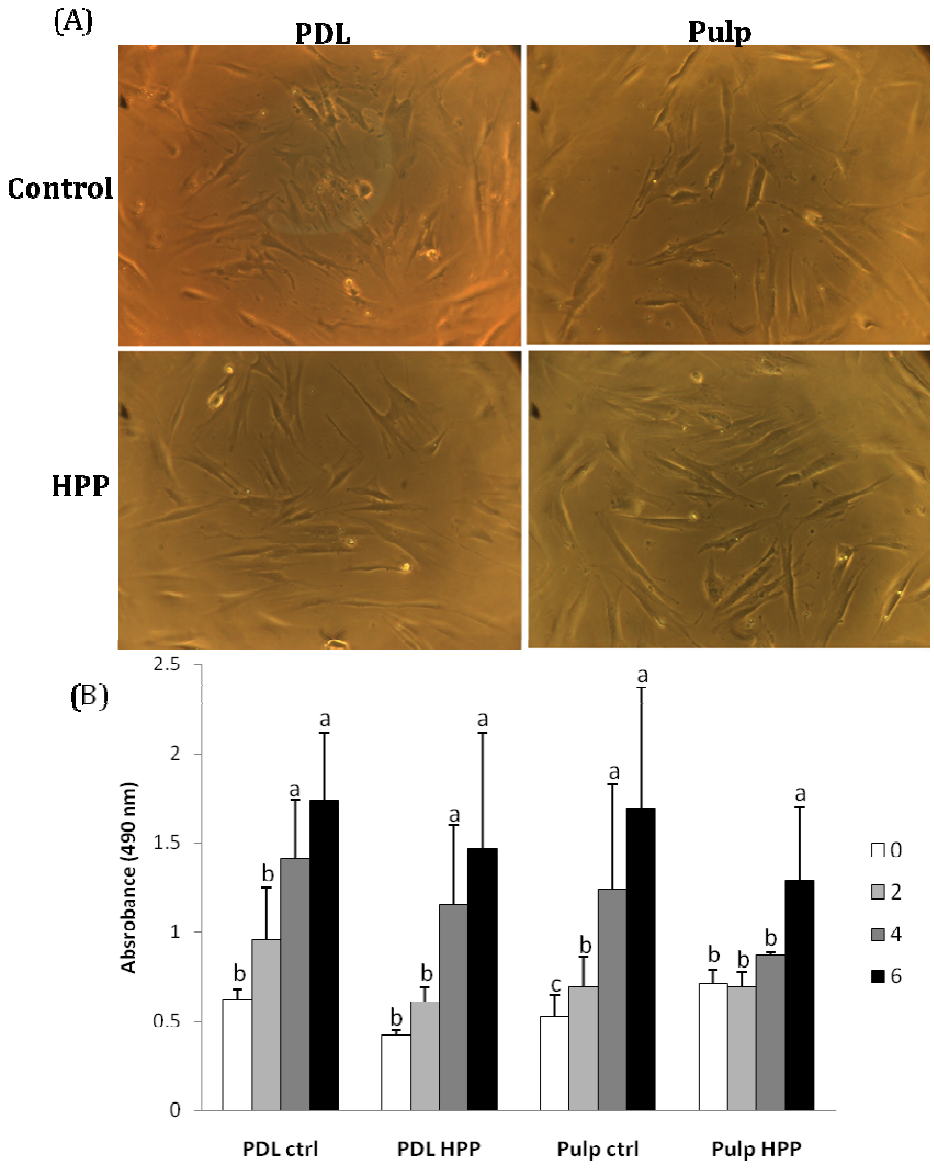

PDLctrl
PDLHPP
Alpl
Ankh
Enpp1
Bsp
Dmp1
Dspp
Mepe
Opn
Pulpctrl
Pulp HPP
Alpl
Ankh
Enpp1
Bsp
Dmp1
Dspp
Mepe
Opn

Figure 4. Expression of P_i/PP_i and mineralization related genes in control and HPP-PDL and pulp cells. Line graphs illustrating mean and SD for the mRNA levels of Alpl, Ankh, Enpp1, Bsp, Dmp1, Dspp, and Mepe, from 5 control and 2 HPP patients over time (1, 5, and 15 days). Values expressed here are from two independent experiments, each conducted in triplicate. + statistically different by the Student t-test (alpha=0.05).

Supplemental Table 1: Primer sequences and Genbank reference number.

Gene	Primer Sequence (5´ → 3´)	Genbank number
Ankh	GAGGTGACAGACATCGTGG CCTTTAAATCAAGGCCTCTTTCATTAC	NM_054027.4
Enpp1	AAATATGCAAGCCCTCTTTGT TTTAGAAGGTGGTTAAGACTTCCATGA	NM_0062208.2
Tnap	CGGGCACCATGAAGGAAAG GCCAGACCAAAGATAGAGTT	NM_000478.4
Dmp1	AGCCATTCTGAGGAAGACGA TGTTGTGATAGGCATCAACTGTTA	NM_004407.3
Dspp	GCATTCAGGGACAAGTAAGCA CTTGGACAACAGCGACATCCT	NM_014208.3
Mepe	ACCTAGAAGGCAAAGATATTCAAACA TTCGCAGTTTCATCCCTAGT	NM_020203.3
Col1	CCCTGGTGCTACTGGTT ACCACGCTGTCCAGCAATA	NM_000088.3
Ocn	AGCTCAATCCGGACTGT GGAAGAGGAAAGAAGGGTGC	NM_199173.3
Gapdh	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	NM_002046.3



Supplemental Figure1: (A) Establishment of PDL and pulp primary cell cultures from normal and HPP patients; (B) Cell growth curve using MTS at 2% FBS. The values are expressed as mean ± SD (absorbance at 490nm) for five control and two HPP patients, in an experiment conducted in triplicate. HPP cells proliferated comparably to control cells. Non-capital letters indicate statistical differences by an intragroup analysis using the Kruskal-Wallis one way ANOVA followed by the Student-Newman-Keuls method (alpha=0.05). There was no significant intergroup difference using the Kruskal-Wallis one way ANOVA within the same experimental period.

CAPÍTULO 3

Phosphate metabolism is a critical factor for periodontal regeneration. An *in vivo* study in mice.

Funding: São Paulo State Research Foundation (FAPESP, Brazil, grant #07/08192-5 (FHNJ), NIH/NIDCR DE15109 (MJS) and NIH FIRCA (5R03TW007590-03) (MJS/FHNJ).

Words in abstract: 247
Total words: 3,936
References: 37
Figures: 5
Running title: Cementum regeneration in *Ank* KO mice.

Summary: In a mouse periodontal fenestration model, *Ank* KO and reduced local levels of PP_i promoted increased cementum regeneration, more rapid and organized apposition, and recapitulation of developmental gene expression and matrix composition.

ABSTRACT

Background: The developing periodontium is sensitive to local levels of phosphate (P_i) and pyrophosphate (PP_i), as demonstrated by cementum phenotypes resulting from loss of function of protein regulators of P_i/PP_i homeostasis. The progressive ankylosis protein (ANK) regulates transport of PP_i, and *Ank* knock-out (KO) mice feature rapidly forming and thick cementum. We hypothesized that, besides affecting cementum formation, decreased extracellular PP_i levels in *Ank* KO mice would also impact on cementum regeneration.

Methods: Periodontal fenestration defects (2mm/1mm/0.5mm) were created on the buccal aspects of mandibular molars in *Ank* KO and wild-type (WT) mice. Mandibles were harvested at 15, and 30 days post-surgery for histology, histomorphometry, evaluation of *in vivo* fluorochrome labeling, and immunohistochemistry (IHC) for proteins including bone

sialoprotein (BSP), osteopontin (OPN), dentin matrix protein 1 (DMP1), and ectonucleotide pyrophosphatase/phosphodiesterase 1 (NPP1).

Results: A greater amount of new cementum was observed for *Ank* KO mice at 15 and 30 days post-surgery ($p<0.05$). Fluorochrome labeling further indicated a higher appositional activity in the defect areas in *Ank* KO vs. controls. At days 15 and 30 during healing, regenerating cementum and associated cells in *Ank* KO recapitulated expression patterns mapped during development, including limited BSP and strong OPN and DMP1 in the cementum matrix, as well as elevated NPP1 in cementoblasts.

Conclusions: Within the limits of the study, these findings suggest that reduced local levels of PP_i can promote increased cementum regeneration. Therefore, local modulation of P_i/PP_i may be a potential therapeutic approach for achieving improved cementum regeneration.

Key words: cementum, phosphate, pyrophosphate, ANK, periodontal regeneration

INTRODUCTION

Predictable regeneration of periodontal tissues following periodontal disease is a major, but currently unrealized, goal of periodontal therapy.[1] In pursuit of treatments that can reverse periodontal destruction, including regeneration of new bone, new cementum, and supportive PDL, attempts have been made to understand cellular and molecular mechanisms and factors regulating formation of these tissues during development and regeneration.[2-4] Toward this goal, we have focused on understanding of the role of phosphate and pyrophosphate in regulation of periodontal tissues, aiming to apply this knowledge towards therapies promoting oral tissue regeneration.

The majority of inorganic phosphate (P_i) in the body exists in the mineralized matrix of bones and teeth as hydroxyapatite (HAP). Maintenance of P_i homeostasis is essential for normal development, maintenance, and repair of teeth and skeletal tissues.[5] Conversely, inorganic pyrophosphate (PP_i) is a potent inhibitor of HAP crystal growth.[6, 7] While P_i balance is governed systemically by the bone-intestine-kidney axis,[5, 8] local PP_i in tissues is primarily controlled by a group of cellular factors. The progressive ankylosis gene (*ANKH* in humans; *Ank* in mouse) encodes a transmembrane protein regulating transport of

intracellular PP_i to the extracellular space.[9-11] Ectonucleotide pyrophosphatase phosphodiesterase 1 (NPP1) is a membrane-bound ectoenzyme that can generate PP_i from extracellular nucleoside triphosphates.[12] Tissue-nonspecific alkaline phosphatase (TNAP) is an ectoenzyme that can hydrolyze PP_i, and is present in high levels in the periodontium as well as mineralized tissues of the bones and teeth.[13-15] These three key regulators work in concert to control local levels of PP_i.

The developing periodontium is especially sensitive to the balance of P_i/PP_i, as demonstrated by cementum phenotypes resulting from loss of function of protein regulators of P_i/PP_i homeostasis.[5, 16-20] Previously, we identified the intriguing tooth phenotype in *Ank* mutant and KO mice, characterized by a marked increase in cervical cementum formation.[16, 18] Mice lacking ANK function feature decreased extracellular PP_i, promoting increased apposition of acellular extrinsic fiber cementum (AEFC). Despite rapid cementogenesis, mechanical properties of cementum were not compromised.[19] Histological and immunohistochemical evidence supported PP_i as a critical molecular regulator of both cementum apposition and composition.

Based on these findings, we hypothesized that modulation of local PP_i levels might be a strategy to encourage cementum regeneration. To that end, we employed the *Ank* KO mouse, with deficient extracellular PP_i and increased cementogenesis, in a proof-of-principle study to analyze tissue repair and regeneration in a periodontal fenestration model.

MATERIALS AND METHODS

Animals

Preparation and genotyping of *Ank* KO mice was described previously.[18, 21] Heterozygote breeding pairs were employed to prepare homozygote *Ank* KO mice and age-matched wild type (WT) controls. Mice were sacrificed by cervical dislocation, in accordance with American Veterinary Medical Association panel on euthanasia, and all procedures were approved by the Institutional Animal Care and Use Committee, University

of Washington (Seattle, WA, USA). Sample size was calculated using previous studies for a power of 0.8 and an alpha of 0.05.

Experimental design and surgical defects

Surgeries were performed on mice at the age of 5 weeks. General anesthesia was delivered by intraperitoneal administration of 130 mg/kg ketamine with 8.8 mg/kg xylasine. A rodent periodontal fenestration defect model was utilized, modified from the method described by King et al.[22] Ten *Ank* KO mice and ten WT control mice were used, with tissues harvested at predetermined time points following surgery: 15 days (n=5 WT and KO), and 30 days (n=5 WT and KO). Periodontal fenestration defects (approximately 2 mm in width, 1 mm in length, and 0.5mm in depth) were created bilaterally at the buccal aspect of the distal root of first and mesial root of second mandibular molars using an operating microscope at 10-40x magnification (Figure 1). Briefly, the superficial bone was removed using a round dental bur (diameter 2 mm), at high speed under saline irrigation, and the distal root of first and mesial root of second mandibular molars were denuded of their periodontal ligament, cementum and superficial dentin using a chisel, avoiding excessive damage to the root that could compromise the endodontic tissues and healing response. Tissues were sutured and buprenorphine (0.05 mg/kg) was given for pain control following surgery, twice daily for 3 days.

Histology

Histological processing was performed as previously described.[18] Briefly, mandibles were removed and sagittally hemisected, with one half prepared for histology and the other used for evaluation of fluorochrome labeling (described below). To prepare samples for use in histology and immunohistochemistry, tissues were fixed in Bouin's solution[§] 24 hrs, thoroughly rinsed with 70% ethanol, and demineralized in AFS solution (10% glacial acetic acid and 1.5% formaldehyde in normal saline solution). Tissues were processed according to standard histological procedures and embedded in paraffin. Serial sections (6 μm) were transversally obtained in an apico-coronal direction of the defect and mounted on charged glass slides. Standard hematoxylin and eosin (H&E) staining was used

for morphological examination. High resolution digital images were captured using a light microscope[‖] with a camera[¶] and software[#] attached.

Histomorphometric analyses

The coronal and apical margins of the defect were identified and 5 sections per specimen representing the middle portion of the defect (≅12μm apart) were selected, and the percentage of defect filling (DF) (%), proportion of mineralized tissue (BD) (%), extension of new cementum (ENC) (μm), proportion of cementum-denuded root coverage by new cementum (PRC) (%) and thickness of new cementum (TNC) (μm) were histomorphometrically assessed using an image analysis system.[**] Measurements from the five sections were averaged for a total of 5 mice each for WT and KO, from 15 and 30 days after surgery. Histomorphometric approaches are summarized in Figure 1. DF and BD were obtained by the point-counting technique.[23] Briefly, a square grid was overlaid on the defect area, and the percentage of defect filling (DF) and mineralized tissue (BD) were determined over the total area of the defect. A mean percentage value was calculated for each animal for statistical analysis. Extension of new cementum (ENC) and thickness of new cementum (TNC) measurements were obtained by linear measurements, whereas the proportion of cementum-denuded root coverage by new cementum (PRC) was obtained by determination of length of new cementum vs. total extent of the instrumented root. TNC measurements were made at fixed distances of 50 μm from mesial and distal edges of pre-existing cementum, and on the central portion of the instrumented root. Histomorphometric parameters were determined by a blinded and calibrated examiner (Intra Class Correlation = 0.92).

Fluorochrome labeling

To analyze the changes in new cementum apposition rate, double fluorescence labeling was performed as described previously,[24] with a minor modification. Briefly, a calcein[††] label (12.5 mg/kg i.p.) was administered to mice 7 days after surgery. Alizarin red[††] label (30 mg/kg i.p.) was administered at 14 days after surgery. Mice were sacrificed at 30 days after surgery, and the mandibles were removed and fixed in 70% ethanol for 48

h. The specimens were dehydrated through a graded series of ethanol (70–100%) and embedded in methyl methacrylate[††] (MMA) without prior decalcification. Fifty-five micrometer-sections were cut using a saw microtome[‡‡] with tungsten carbide knife.[‡‡] The unstained sections were viewed under epifluorescent illumination using a microscope,[‖] interfaced with software[#] and camera.[¶]

Picrosirius red stain for collagen

Tissues processed for histology were stained with a picrosirius red staining kit[§§] according to manufacturer directions. Briefly, slides were immersed in 0.2% phosphomolybdic acid hydrate, rinsed in water, incubated in direct red 80 for 60 min, then 0.01 N HCl solution for an additional 2 min. Samples were rinsed in 75% ethanol for 45 sec, then dehydrated in xylene, cleared and mounted with coverslips. Digital images were captured with a light microscope[‖‖] fitted with a light polarizer, with a digital camera.[¶¶]

Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described.[18, 25] Mouse mandible tissues were deparaffinized in xylene and rehydrated using decreased graded dilutions of ethanol. Tissues were permeabilized in acetone at -20°C for 10 min, and endogenous peroxidase was quenched by incubation in 3% H_20_2 in methanol solution for 1 hr. Primary antibodies were used with biotinylated secondary antibodies[##] against rabbit or goat primary antibodies, as appropriate, and slides were developed using a 3-amino-9-ethylcarbazole (AEC) substrate kit.[##] Positive controls included staining in WT tissues, where immunolocalization of target proteins has been well characterized. Negative controls were performed lacking primary antibody. Primary antibodies used to react with mouse targets of interest included: rabbit anti-recombinant mouse bone sialoprotein (BSP), (a gift from Dr. Renny Franceschi, University of Michigan); rabbit anti-rat dentin matrix protein-1[***] (DMP1) raised against an N-terminal (90-111) portion of DMP1; goat anti-human NPP1;[†††] LF-175 rabbit anti-mouse osteopontin (OPN) (Dr. Larry Fisher, NIDCR).[26] IHC for each target protein was performed in sections from at least three WT and *Ank* KO

animals for each age, with representative staining for selected antibodies shown in the Results section.

Statistical analysis

The null hypothesis of no difference between WT and KO groups regarding the evaluated parameters was tested by the parametric Student t-test and non-parametric Mann-Whitney test for linear and percentual measurements, respectively with $\alpha=0.05$.

RESULTS

Histological analysis of defect healing

The surgical wounds healed well with no signs of infection and no observable ingrowth of oral or skin epithelium into the defect. After surgeries, animals continued to feed normally and gain weight.

Histological sections from day 15 indicated newly formed bone and fibrous tissue scattered within the defect area in both *Ank* WT and KO mice (Figure 2A-D). The defect area could be identified by the reversal line, which clearly divided pre-existing and new-formed bone. New cementum, when observed, occurred adjacent to pre-existing cementum in the form of spicules or projections, being predominantly of cellular type and more frequently present in *Ank* KO mice.

By day 30, the overlying buccal alveolar bone was restored in both *Ank* WT and KO mice. In *Ank* KO at this time point, new cementum formation was more consistently present on cut root surfaces, compared to WT controls (Figure 2E-H). Neither root resorption nor ankylosis was observed in any of the groups and times points.

Loss of ANK induced more rapid and increased cementum regeneration

Histomorphometry was employed to detect and quantify dimensions of cementum regeneration in *Ank* KO and WT, as well as determine if surrounding periodontal tissues were altered during defect repair. Defect filling was assessed at days 15 and 30 for unfilled space, soft tissue, and new bone, and no major differences were detected in % in

Ank KO vs. WT controls (Figure 3A). Within the limits of analysis, no differences were observed in the proportion of mineralized (BD) in the new-formed bone in WT vs. KO mice (Figure 3B). Linear measurements for extension of new cementum (ENC) formation showed significantly greater extension of new cementum formation for the KO animals versus WT at 15 and 30 days after the defect creation ($p<0.05$), approximately 4-fold at day 15 and 2-fold at day 30 (Figure 3C). Furthermore, the proportion of cementum-denuded root coverage by new cementum was significantly greater on the *Ank* KO cut root surface compared with WT, more than 2.5-fold at day 15 and more than 1.7-fold at day 30 (Figure 3D). Comparison of thickness of new cementum (TNC) in *Ank* KO vs. WT revealed that KO mice featured significantly greater deposition of new cementum along the cut root surface, a trend increasing with time (Figure 3E). Therefore, increased cementogenesis was detected by 15 days post-surgery, though the effect was even greater by day 30.

Progress of new cementum apposition was observed using vital fluorochrome labeling, with fluorescent microscopy performed on tissues harvested 30 days post-surgery. In regions of pre-existing cementum and bone outside of the defect area, calcein dye (green) injected at 7 days after surgery was observed with similar intensity in *Ank* KO and WT. This dye, administered relatively early in the healing process, provided no evidence for cementum deposition in the defect region at 7 days post-surgery, for both WT and KO (Figure 4A-B). The second fluorochrome, alizarin red (red) dye, injected at 14 days after surgery was observed in both WT and *Ank* KO, suggesting some mineral deposition for both groups by this stage in healing. However, fluorochrome labeling lines in *Ank* KO cementum appeared consistently thicker and more concentrated than controls, indicating more rapid mineral deposition in the defect areas in *Ank* KO vs. controls.

Picrosirius red staining observed under polarized light enhances the natural birefringence of collagen, allowing assessment of collagen fiber organization.[27] At 30 days post-surgery in WT and KO, some PDL collagen fibers were observed to be parallel to the healing root surface. However, for both WT and KO, some organized and functionally oriented periodontal ligament fibers were also observed (Figure 4C-D).

Cementum regeneration in *Ank* KO recapitulates developmental changes in extracellular matrix proteins

To better understand the nature of the regenerated cementum and the mechanisms contributing to cementum regeneration in WT and *Ank* KO, immunohistochemistry (IHC) was used. We analyzed expression of selected cementum, bone, and dentin markers at days 15 and 30 during periodontal defect repair. Developmental expression of bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein 1 (DMP1), and ectonucleotide pyrophosphatase phosphodiesterase 1 (NPP1) was previously mapped during tooth root formation in *Ank* KO mice,[18] and expression during periodontal healing will be considered in the context of those findings.

BSP is present in bone and cementum extracellular matrix, and immunolocalizes as a concentrated band defining the acellular cementum layer. Staining of BSP in pre-existing bone was similar between WT and *Ank* KO. In WT, cementum was labeled by strong, even staining, while BSP presence was more diffuse in KO (Figure 5A-D). By 15 days post-surgery, BSP protein expression was robust in the new bone of WT and KO mice (Figure 5A-B). Little BSP was immunolocalized to the healing root surface at this stage. By day 30, the thin layer of new cementum on WT roots featured strong BSP staining reminiscent of the original AEFC layer. In KO, the regenerated cementum layer was BSP positive, though not uniformly so (Figure 5C-D).

Osteopontin (OPN) is also present in bone and cementum, as well as in the soft tissue of the PDL. OPN was strongly immunopositive in bone and cementum of both WT and KO (Figure 5E-H). By day 15, OPN was localized to the healing root surfaces in both WT and *Ank* KO molars. Ank KO samples featured a larger, generally more intense zone of OPN-positive matrix and cells on the defect surface, as well as more intense OPN in the adjacent PDL. OPN staining was similar in WT vs. KO at 30 days, except for notably increased OPN in *Ank* KO PDL.

Dentin matrix protein 1 (DMP1) localized primarily to bone matrix around osteocytes, with lower levels of staining apparent in dentinal tubules (Figure 5I-L). WT acellular cementum did not stain positively for DMP1. At day 15, there was no detectable DMP1 staining in healing sites either group. By day 30, DMP1 localized strongly to matrix

and cells in *Ank* KO repair cementum, while in WT, DMP1 stained weakly and primarily around cementocytes.

We have identified low level expression of NPP1 in odontoblasts and osteoblasts, whereas cementoblasts associated with AEFC expressed higher levels of NPP1.[18] By day 15, no NPP1 expression could be detected at the healing site in WT samples (Figure 5M). In *Ank* KO at 15 days, NPP1 expression was strong in cementoblasts associated pre-existing cementum, but little expression was detected in the healing defect except in a small number of osteoblasts (Figure 5N). By day 30, NPP1 expression in WT was detectable in some cementoblasts and osteoblasts on regenerated tissues (Figure 5O). Osteoblast NPP1 expression in *Ank* KO was similar to WT, though cementoblasts at the healing site exhibited increased NPP1 at this time point. An additional extracellular matrix protein assayed by IHC, periostin, was found to be no different in *Ank* KO vs. WT (data not shown).

DISCUSSION

This is the first study to assess wound healing in mice using an isolated uniform periodontal wound healing model, while maintaining functional occlusion, without oral bacterial contamination or ingrowth of gingival epithelium into the wound space. This periodontal defect model, used previously by our group and others, has proven to be a predictable and reliable model for studying periodontal healing in rats.[22, 23, 28, 29] Although this model may not represent a critical-size defect for periodontal regeneration in mice, it serves as a reasonable screening model to examine wound-healing kinetics in the periodontium. Importantly, the experimental design also allowed us to ask the question whether a loss of gene function affecting cementum formation could be exploited to test the hypothesis that regeneration would recapitulate development.

In this study, we compared periodontal regeneration in WT controls to the *Ank* KO mice, a model with reduced tissue PP_i that exhibits increased cementogenesis and hypercementosis.[18] Based on the developmental phenotype, we hypothesized that reduced extracellular PP_i levels would encourage greater cementum regeneration. Remarkably, we

found that *Ank* KO mice featured significantly greater new-formed cementum vs. controls, at 15 and 30 days post-surgery ($p<0.05$). Further, vital fluorochrome labeling indicated more organized mineral deposition on the root surface in the defect areas in *Ank* KO vs. controls. At days 15 and 30 during healing, regenerating cementum and associated cells in *Ank* KO recapitulated expression patterns mapped during cementogenesis, including limited BSP and strong OPN and DMP1 in the cementum matrix, as well as elevated NPP1 in associated cementoblasts. Within the limits of the study, we found that reduced local levels of PP_i promoted increased cementum regeneration, suggesting that a novel strategy to reduce PP_i or modulate P_i/PP_i in the periodontal region may encourage greater and more predictable cementum regeneration.

Previous studies in mouse models and human patients have identified PP_i as an important regulator of the acellular cementum development, and this has provided the rationale for our current approach to cementum regeneration. Local cell and tissue level concentrations of PP_i are controlled primarily by a number of regulatory enzymes and transporters. Tissue nonspecific alkaline phosphatase (TNAP) is an ectoenzyme that hydrolyzes PP_i and is important for bone mineralization, as well as being active in the periodontal region.[13-15] The condition hypophosphatasia (HPP), which causes rickets, and osteomalacia, results from mutations in the gene for TNAP.[15, 30-32] HPP provided the first suggestion of the sensitivity of cementum to PP_i homeostasis because in both humans and mice, TNAP deficiency causes cementum aplasia or severe hypoplasia, poor periodontal attachment, and exfoliation of teeth.[15, 17, 20]

The progressive ankylosis protein (ANK) is a membrane spanning protein that regulates transport of intracellular PP_i to the extracellular space.[9-11] Another PP_i regulator, ectonucleotide pyrophosphatase phosphodiesterase 1 (NPP1), also increases extracellular PP_i, but by enzymatic hydrolysis of nucleoside triphosphates. ANK and NPP1 offset TNAP activity by increasing extracellular concentrations of the mineralization inhibitor, PP_i.[12] We have described the developmental tooth phenotype in mice lacking ANK or NPP1 function, characterized by a marked increase in cementum formation, while other tooth tissues (e.g. PDL, dentin, and alveolar bone) appeared unaffected.[16, 18, 19] While ANK, NPP1, and TNAP are widely expressed, we and others have identified increased expression and/or activity in

PDL vs. the pulp/dentin complex, highlighting the importance of PP_i metabolism in the Periodontia.[17, 33]

Here, we observed that loss of ANK function encouraged more robust cementum regeneration, resulting in significantly increased new cementum coverage of the root and thicker cementum in KO vs. control mice. In our developmental studies, we found that PP_i governs appositional rate of the acellular cementum.[16, 18] We reasoned that in the context of the fenestration defect, provided perio-progenitor cells were available to repopulate the defect region and differentiate to a cementoblast phenotype, that lack of ANK function and the associated PP_i deficiency would similarly create an environment conducive to cementogenesis. Histological observations, histomorphometry, and observation of more organized vital dye labeling in *Ank* KO tissues support this assertion and our hypothesis. Some functionally oriented PDL fibers were identified on healing defect surfaces in KO as well as WT mice. Organization and insertion of PDL fibers in conjunction with rapid and predictable cementum formation provide an opportunity for true periodontal regeneration, i.e. a restoration of the structure and function of the periodontium.[1]

We analyzed expression of selected cementum, bone, and dentin markers during periodontal defect repair to better understand the nature of the regenerated cementum in WT and *Ank* KO, and to parallel mapping of these same factors in cementum development.[18] New cementum formation in WT defects was marked by robust labeling of BSP and OPN, classic markers for acellular cementum.[34-36] In defects in *Ank* KO mice, new cementum also mirrored developmental patterns, including limited BSP, strong OPN and DMP1 in the cementum matrix, and heightened NPP1 in associated cementoblast cells. We have demonstrated that these changes in AEFC composition in *Ank* KO teeth are the results of rapid and increased cementum apposition; as cementum thickness increases, BSP becomes limited, cementoblasts increase expression of OPN and NPP1 in response to increased apposition, and DMP1 is increased in the matrix around embedded cells (cementocytes).[18] We report here for the first time that loss of ANK caused altered cementoblast gene expression during defect healing, and importantly show that these changes recapitulate cementum development in this model.

CONCLUSIONS

These results serve as validation for a novel proof-of-principle concept for periodontal regeneration. Within the limits of the study, we found that reduced local levels of PP_i promoted increased cementum regeneration, suggesting that a novel strategy to reduce PP_i or modulate P_i/PP_i in the periodontal region may encourage greater and more predictable cementum regeneration. While more work must be done to better understand other mechanistic aspects of cementum regeneration, e.g. factors encouraging PDL fiber insertion in regenerating cementum in order to make a strong interface, the possibility for more rapid and predictable cementum formation is an encouraging step forward.

FOOTNOTES

§ Electron Microscopy Sciences, Fort Washington, PA, USA

‖ Nikon Eclipse E400, Nikon Instruments Inc, Melville, NY, USA

¶ SPOT CCD camera, Diagnostic Instruments, Sterling Heights, MI, USA

Metavue, Molecular Devices, Sunnyvale, CA, USA

** Image-Pro Plus 4.5; Media Cybernetics Inc., Silver Spring, MD, USA

†† Sigma-Aldrich, Saint Louis, MO, USA

‡‡ Leica SM 2500, Leica Microsystems Nussloch GmbH, Nussloch, Germany.

§§ Polysciences, Inc., Warrington, PA, USA

‖‖ Nikon OptiPhot-2, Nikon Instruments Inc, Melville, NY, USA

¶¶ Canon EOS 5D Mark II, Canon U.S.A., Inc., Lake Success, NY, USA

Vector Laboratories, Inc., Burlingame, CA, USA.

*** Takara Bio, Inc., Shiga, Japan

††† AbCam, Inc., Cambridge, MA, USA

ACKNOWLEDGMENTS

These studies were supported by São Paulo State Research Foundation (FAPESP, São Paulo, SP, Brazil) grant 08/00534-7 (FHNJ), NIH FIRCA (5R03TW007590-03) (MJS/FHNJ) and NIH/NIDCR grant DE15109 (MJS). The authors declare no conflicts of interest in these studies. The authors thank Jirawan Wade for preparation of plastic embedded sections. We thank Dr. Larry Fisher and Dr. Renny Franceschi for generously provided antibodies for these studies.

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FIGURES

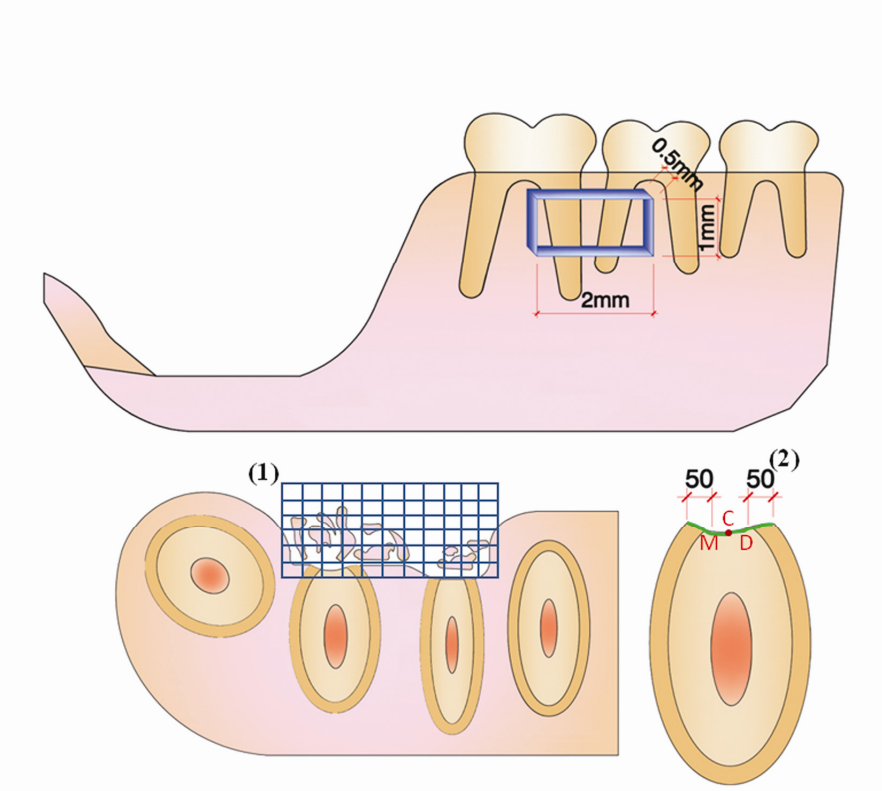

Figure 1. Schematic illustration of histomorphometry. Histomorphometry was used to assess percentage of defect filling (DF) (%), proportion of mineralized tissue (BD) (%), extension of new cementum (ENC) (µm), proportion of cementum-denuded root coverage by new cementum (PRC) (%) and thickness of new cementum (TNC) (µm). DF and BD were measured using a square grid (1) overlaid on the defect area. ENC was obtained by linear measurement of newly formed cementum, and PRC determined by the proportion of newly formed cementum covering the total extent of the instrumented root (green line). TNC measurements were made at fixed distances of 50 µm from mesial (M) and distal (D) side of pre-existing cementum, and on the central (C) region of the instrumented root (2).

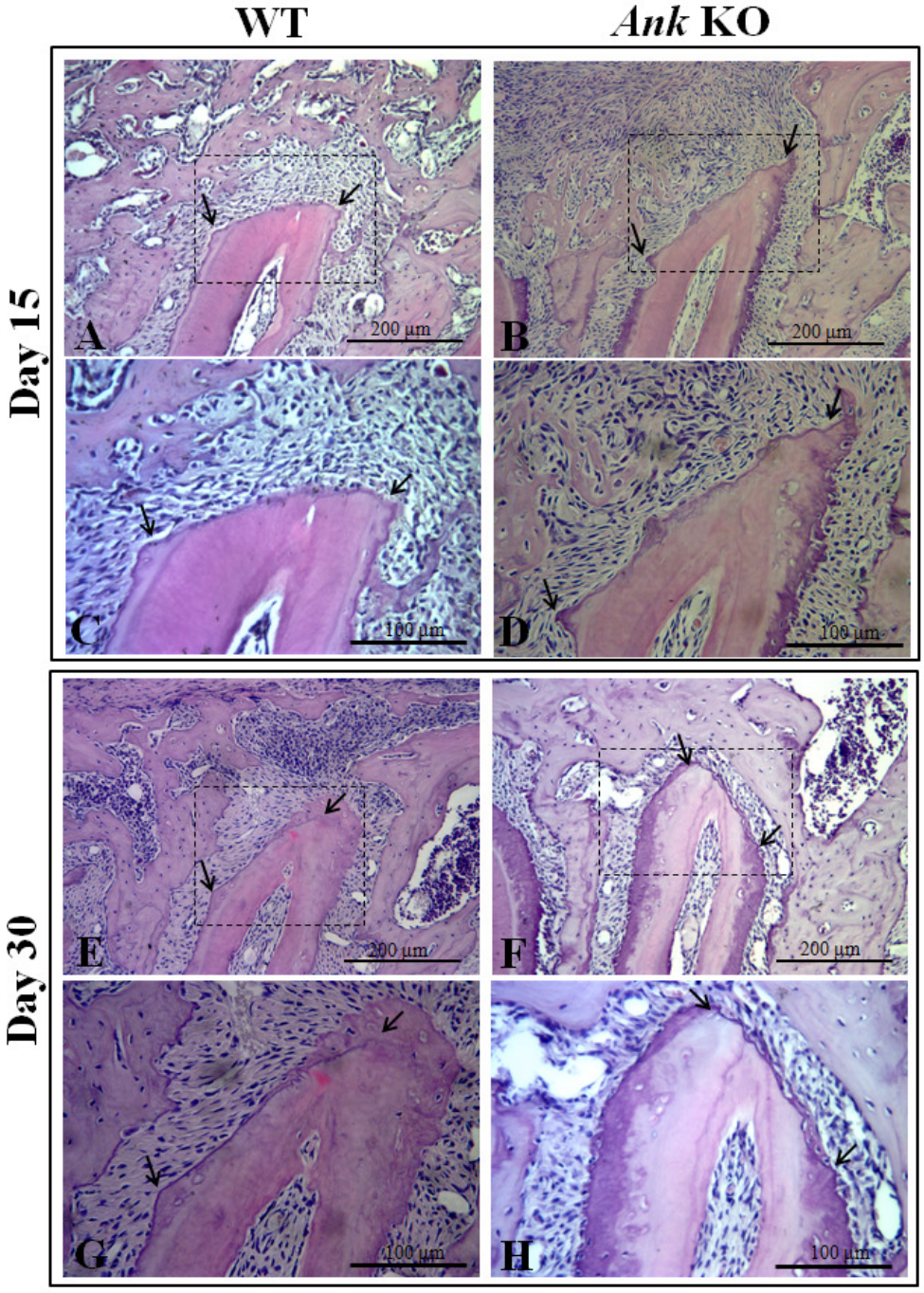

Figure 2. Histological analysis of periodontal defect healing in WT and *Ank* KO. Representative H&E stained slides from WT and Ank KO mice at 15, and 30 days post-surgery. Arrows indicate margins of pre-existing cementum and rectangles define the root instrumented area used for linear measurements that is also presented in higher

magnification. At 15 days post-surgery, both WT and *Ank* KO roots featured newly formed bone and fibrous tissue. New cementum, predominantly of the cellular type, was more frequently observed in *Ank* KO mice. By day 30, restoration of overlying alveolar bone was observed in defects in both WT and *Ank* KO mice. In *Ank* KO at this time point, new cementum formation was more consistently present on all cut root surfaces, compared to WT controls.

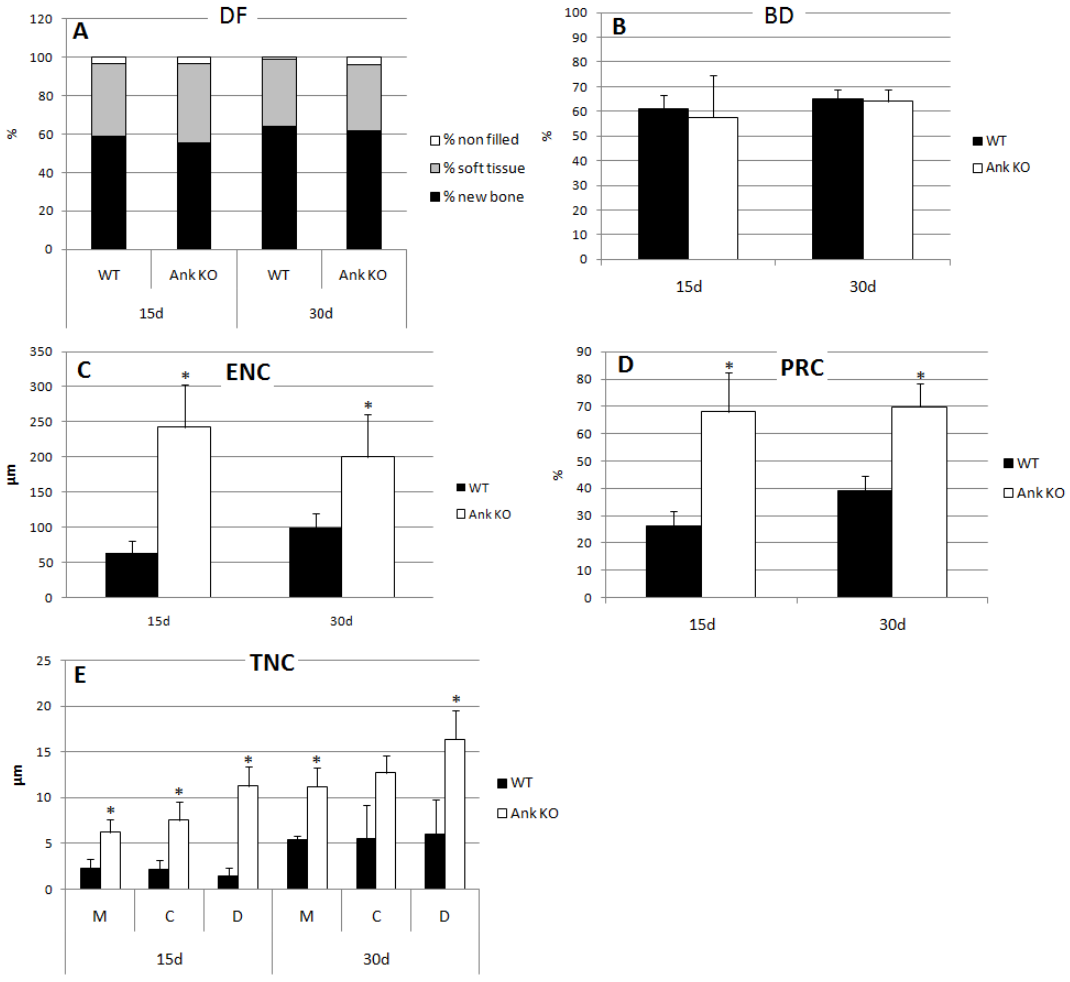

Figure 3. Histomorphometry confirmed loss of ANK induced more rapid and increased cementum regeneration. Histomorphometric approaches are described in the Methods and Figure 1. **(A)** No major differences were detected in defect filling parameters in *Ank* KO vs. WT at days 15 and 30. **(B)** New bone density (BD) was not different in *Ank*

KO vs. WT at both time points. **(C)** Extension of new cementum (ENC) and **(D)** the proportion of root coverage by new cementum (PRC) were significantly increased in *Ank* KO vs. WT at 15 and 30 days. **(E)** Thickness of new cementum (TNC) measured at mesial side of the root (M), center of the root (C), and distal side of the root (D) was greater for all parameters in *Ank* KO vs. WT. * $p<0.05$.

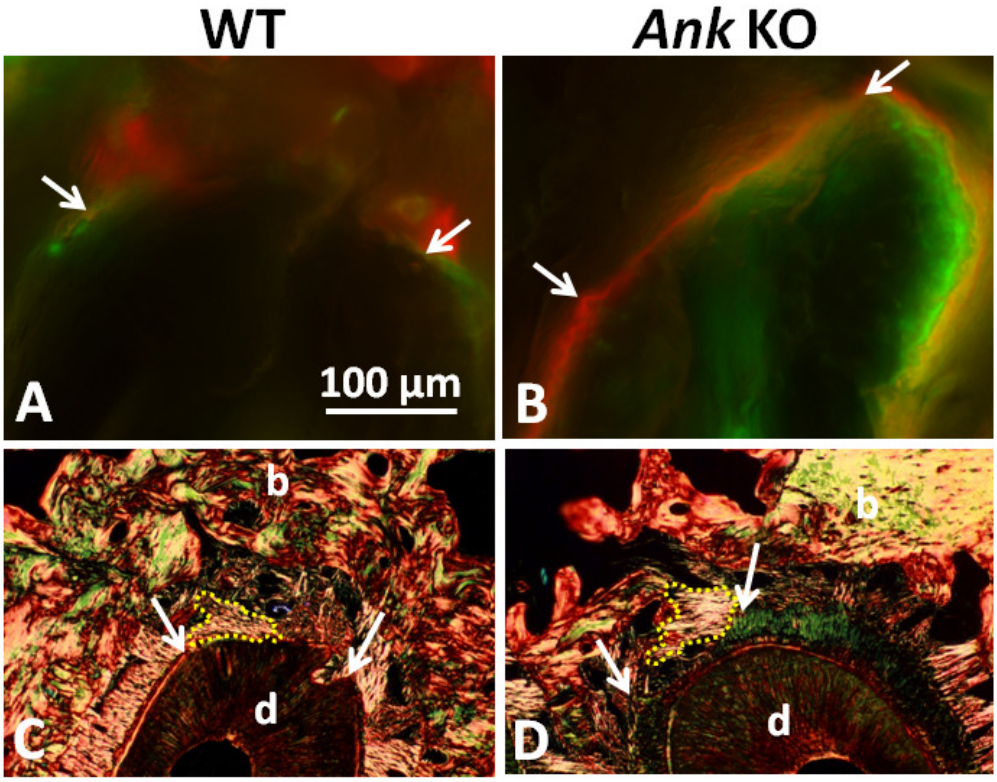

Figure 4. Labeling of new cementum apposition and periodontal ligament fiber organization. (A-B) In regions of pre-existing cementum and bone away from the defect area, calcein dye (green) injected 7 days after surgery was observed with similar intensity in WT and *Ank* KO. Alizarin labeling (red) injected at day 14 was observed in WT and KO, though staining in *Ank* KO appear thicker and more concentrated than in controls. **(C-D)** Regions of organized and functionally oriented PDL fibers (yellow outline) were identified in both WT and KO, in addition to more parallel oriented fibers. White arrows in A-D indicate the defect margins. Abbreviations: b=bone, d=dentin.

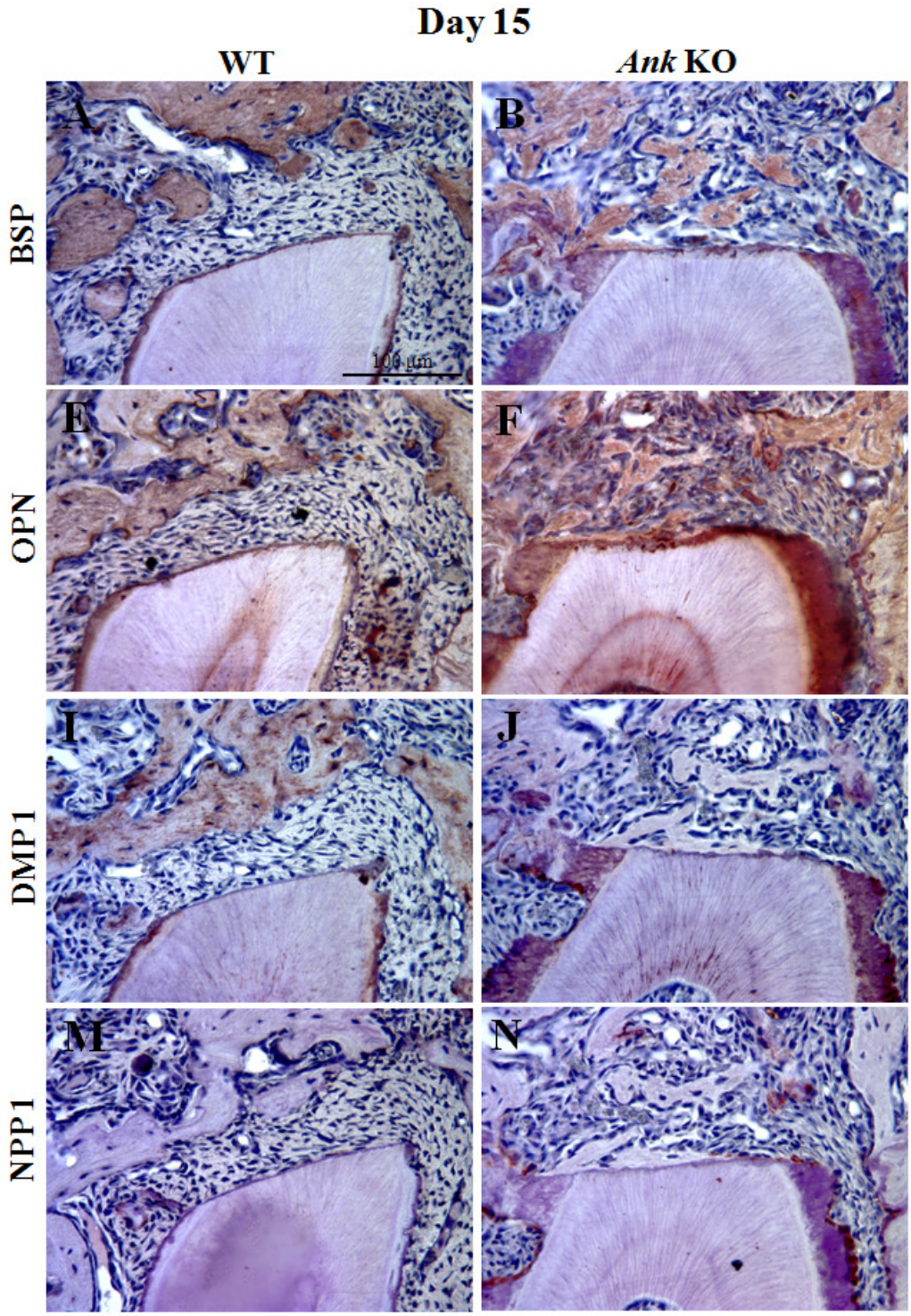

Figure 5: (first part)

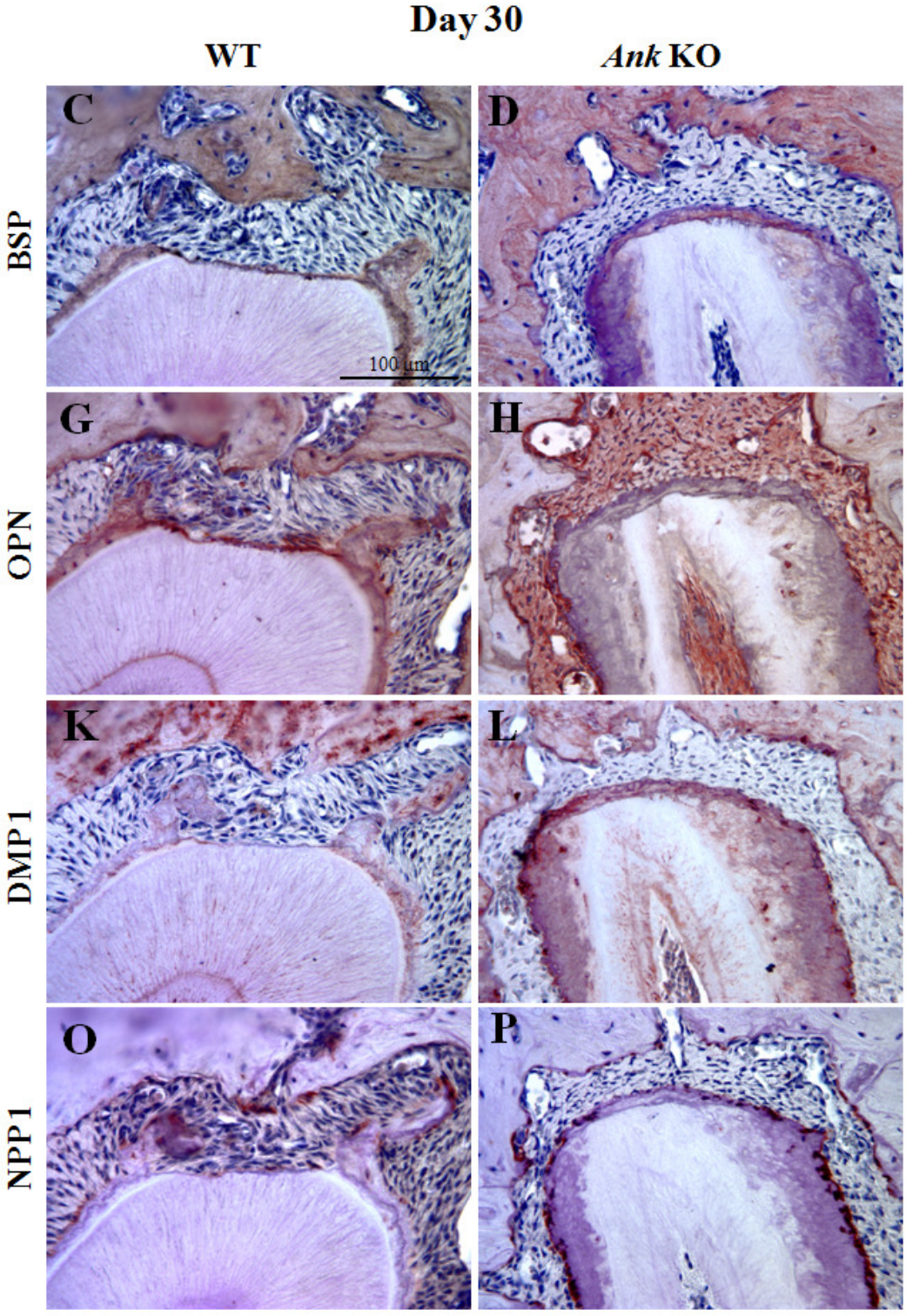
Day 30
WT
Ank KO
BSP
OPN
DMP1
NPP1
C
D
G
H
K
L
O
P
100 µm

Figure 5 (cont.): Expression of mineralized tissue markers during cementum regeneration. Histological sections from days 15 and 30 were used for IHC. **(A-D) Bone sialoprotein (BSP):** By day 15, BSP protein expression was robust in the new bone of WT and KO mice, though little BSP was localized to the healing root surface. By day 30, the thin layer of new cementum on WT roots featured strong BSP staining, while new cementum in KO was BSP positive, though not uniformly so. **(E-H) Osteopontin (OPN):** OPN was strongly positive in bone and cementum of WT and KO. By day 15, OPN was localized to the healing root surfaces in both WT and *Ank* KO molars, though more intense OPN staining was consistently identified in KO new cementum and PDL. OPN staining was similar in WT vs. KO at 30 days, except for notably increased OPN in *Ank* KO PDL. **(I-L) Dentin matrix protein 1 (DMP1):** DMP1 localized primarily to bone matrix around osteocytes, with lower levels of staining apparent in dentinal tubules. WT acellular cementum did not stain positively for DMP1. At day 15, there was no detectable DMP1 staining in healing sites either group. By day 30, DMP1 localized strongly to matrix and cells in *Ank* KO repair cementum, while in WT, DMP1 stained weakly and primarily around cementocytes. **(M-P) Ectonucleotide pyrophosphatase phosphodiesterase 1 (NPP1):** By day 15, no NPP1 expression could be detected at the healing site in WT samples, though in *Ank* KO, NPP1 expression was strong in cementoblasts associated pre-existing cementum. By day 30, NPP1 was detectable in some WT cementoblasts and osteoblasts on regenerated tissues. Osteoblast NPP1 expression in *Ank* KO was similar to WT, though cementoblasts at the healing site exhibited increased NPP1.

CONCLUSÃO

Dentro dos limites do presente estudo, pode-se concluir que:

i) A perda prematura de dentes decíduos na ausência de desordens esqueléticas pode servir como um sinal inicial crítico para o diagnóstico de odonto-HPP e outros subtipos;

ii) Os dados sugerem que há uma diferença importante no comportamento *in vitro* entre as células controle e HPP, incluindo a expressão basal dos genes relacionados ao cemento bem como suas capacidades de promoverem a formação de minerais. Estes resultados podem trazer novas perspectivas ao tratamento de pacientes com deficiências no metabolismo do fosfato.

iii) Dentro dos limites do estudo, os achados sugerem que níveis reduzidos de PP_i local pode promover um aumento da regeneração do cemento. Portanto, a modulação entre P_i/PP_i pode ser uma potente abordagem terapêutica para alcançar melhoras na regeneração do cemento.

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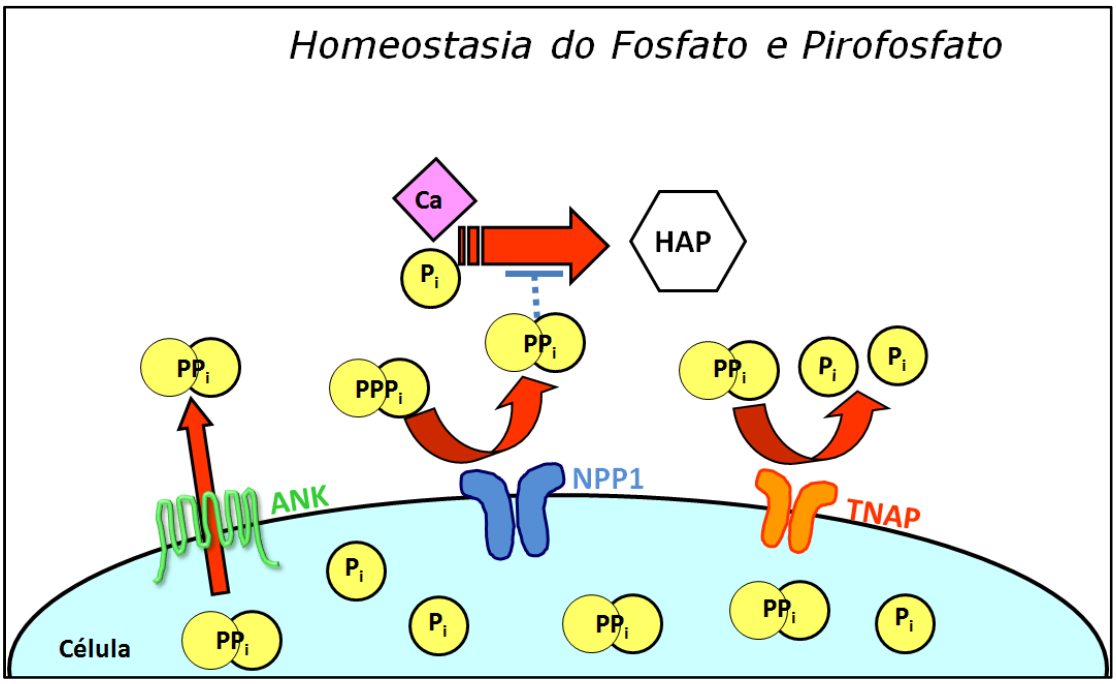
Anexo 1:



HAP: hidroxiapatita
Ca: Cálcio
PPP_i:adenosina trifosfato
PP_i: pirofosfato inorgânico
P_i: fosfato inorgânico
ANK: proteína de anquilose
NNP1: glicoproteína 1 (*ectonucleotidepyrophosphatase-phosphodiesterase1)*
TNAP: fosfatase alcalina

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Regulação gênica de células da polpa e ligamento periodontal em indivíduos com hipofosfatasia**", protocolo nº **065/2005**, dos pesquisadores **FRANCISCO HUMBERTO NOCITI JUNIOR**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 07/07/2005.

The Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas, certify that project "**Regulation of dentin-pulp and periodontal ligament cells in individuals with hypophosphatasia**", register number **065/2005**, of **FRANCISCO HUMBERTO NOCITI JUNIOR**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for researching in human subjects and was approved by this committee at 07/07/2005.

Cinthia Pereira Machado Tabchoury
Secretária
CEP/FOP/UNICAMP

Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

Anexo 3:

11/07/2006 16:19 0612266453 CONEP PAGE 01

MINISTÉRIO DA SAÚDE
Conselho Nacional de Saúde
Comissão Nacional de Ética em Pesquisa - CONEP

065/2005

PARECER Nº 1156/2006

Registro CONEP: 13114 (Este nº deve ser citado nas correspondências referentes a este projeto)

CAAE – 0062.0.167.000-05 **Processo nº 25000.098177/2006-62**
Projeto de Pesquisa: *"Regulação gênica de células da polpa e ligamento periodontal em indivíduos com hipofosfatasia ."*
Pesquisador Responsável: Dr. Francisco Humberto Nociti Júnior.
Instituição: Universidade Estadual de Campinas /Faculdade de Odontologia de Piracicaba.
CEP de origem: 005/2006
Área Temática Especial: Cooperação estrangeira
Patrocinador: FAPESP

Ao se proceder à análise do projeto de pesquisa em questão, em resposta ao Parecer CONEP nº 870/2006, cabem as seguintes considerações:

a) O pesquisador informou que a etapa do projeto caracterizada como Grupo I só iniciará após a aprovação da CONEP.
b) O país de origem é o Brasil e a colaboração estrangeira se refere à execução de análises laboratoriais. A propriedade das informações será exclusiva dos brasileiros.
c) O Termo de Consentimento Livre e Esclarecido foi refeito com adequações da apresentação.
d) As informações enviadas atendem aos aspectos fundamentais da Res. CNS 196/96 sobre diretrizes e normas regulamentadoras de pesquisas envolvendo seres humanos.
e) O projeto foi aprovado pelo Comitê de Ética em Pesquisa – CEP da instituição supracitada.

Diante do exposto, a Comissão Nacional de Ética em Pesquisa – CONEP, de acordo com as atribuições definidas na Res. CNS 196/96, manifesta-se pela aprovação do projeto de pesquisa proposto.

Situação: **Protocolo aprovado.**

Brasília, 3 de novembro de 2006.

WILLIAM SAAD HOSSNE
Coordenador da CONEP/CNS/MS

Anexo 4:

UNICAMP

Universidade Estadual de Campinas
Instituto de Biologia

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **976-1**, sobre **"MODULAÇÃO DE FOSFATO NOS TECIDOS DENTAIS"** sob a responsabilidade do **Prof. Dr. Francisco Humberto Nociti Junior** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 07 de março de 2006.

CERTIFICATE

We certify that the protocol nº **976-1**, entitled **"PHOSPHATE MODULATION OF DENTAL TISSUES"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on March 7, 2006.

Campinas, 07 de março de 2006.

Profa. Dra. Ana Maria A. Guaraldo
Presidente - CEEA/IB/UNICAMP
CEEA/IB/UNICAMP

Fátima Alonso
Secretária

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