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EFEITO DA ADMINISTRAÇÃO INTERMITENTE DO HORMÔNIO PARATIREÓIDEO (PTH) NA FORMAÇÃO DE DENTINA EM CAMUNDONGOS

Dissertação de mestrado apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, como parte dos requisitos para obtenção do Título de Mestre em Biologia Buco-Dental, na área de concentração em Histologia e Embriologia.

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RESUMO

A dentina, assim como o osso, é um tecido mineralizado de natureza conjuntiva. Embora já tenha sido relatado que o hormônio paratireóideo (PTH), um dos reguladores da homeostasia do cálcio, participe do processo de formação de dentina, a maioria dos trabalhos envolvendo este hormônio é relacionado ao tecido ósseo. Tal hormônio, que fisiologicamente promove a reabsorção óssea, quando administrado de forma intermitente, pode promover anabolismo ósseo, sendo hoje um dos tratamentos para osteoporose. Por serem ainda pouco conhecidas as funções do PTH na dentinogênese, o objetivo deste estudo foi investigar os efeitos da administração intermitente de PTH sobre a taxa de aposição e características estruturais da dentina em incisivos de camundongos. Para tanto, camundongos A/J Unib foram divididos em quatro grupos: C6, T6, C10 e T10 (n=10). Os animais dos grupos T6 e T10 receberam injeções subcutâneas de 40µg/Kg de hPTH(1-34), durante 6 e 10 dias respectivamente; os dos grupos C6 e C10 receberam injeções subcutâneas do veículo do PTH, também durante 6 e 10 dias respectivamente. Para delimitação da dentina formada no período experimental, os animais dos grupos T6 e C6 receberam injeções intraperitoneais de dois marcadores fluorescentes. Ao término do período experimental, o sangue dos animais dos grupos C6 e T6 foram coletados para quantificação de fosfatase alcalina e, suas hemimandíbulas esquerdas foram removidas para medição das taxas de aposição dentinária. Os animais dos grupos T10 e C10 foram sacrificados e as hemimandíbulas, direita e esquerda, foram processadas para os testes de microdureza knoop da dentina e conteúdo elementar (% de átomos) de cálcio (Ca), fósforo (P), oxigênio (O) e magnésio (Mg) na dentina peritubular e na dentina intertubular por meio da microanálise de energia dispersiva de raios-X (EDX). Após análise estatística pelo teste T de *student*, verificou-se um aumento significativo de 5% na taxa de aposição de dentina e a análise enzimática detectou um aumento significativo de 25% de fosfatase alcalina no grupo T6 em relação ao grupo C6. Os animais do grupo T10 apresentaram maior (11%) microdureza na dentina do que os animais do grupo C10. A microanálise por EDX demonstrou que

o tratamento com PTH (T10) levou ao aumento do conteúdo (% de átomos) de P (23%) e Ca (53%), bem como da relação Ca/P (24%) na dentina peritubular, em relação ao grupo C10 ($p<0.01$). Estes resultados indicam que a administração intermitente de PTH, teve um efeito anabólico na formação de dentina em camundongos, acompanhado de significativas alterações estruturais na dentina formada.

Palavras-chave: hormônio paratireóideo, dentina, microdureza knoop, EDX, camundongos.

ABSTRACT

Parathyroid hormone (PTH) has been widely studied, especially in the treatment of osteoporosis, since when administered intermittently promotes bone anabolism. Dentin is a mineralized tissue that share certain similarities to the bone. However, the role of this hormone on dentin formation is poorly known. The purpose of this study was to investigate the effects of intermittent PTH administration on the apposition rate and structural features of dentin from mouse incisors. Forty young male mice A / J Unib were divided into four groups (n = 10): T6 and T10 = animals received subcutaneous injections of 40µg/Kg of hPTH (1-34) diluted in 0.01% acetic acid for 6 and 10 days respectively; C6 and C10 = animals received subcutaneous injections of PTH vehicle, also for 6 and 10 days, which served as control. The animals of C6 and T6 groups received intraperitoneal injections of fluorescent markers for delimitation of dentin formed during the experimental period. Dentin apposition rates, measured by fluorescence microscopy, and the detection of alkaline phosphatase (ALP) plasma levels were evaluated in the animals of T6 and C6 groups. Knoop microhardness testing and element content measurements in atom % of calcium (Ca), phosphorus (P), oxygen (O), and magnesium (Mg) in the peritubular and intertubular dentin by Energy Dispersive X-ray (EDX) microanalysis via Scanning Electron Microscopy (SEM) were performed in the animals of T10 and C10 groups. Statistical analysis of all tests was completed using unpaired Student's t test (two-tailed). Statistical significance limit was set at 5%, ($p<0.05$). Histometrical analysis by fluorecence microscopy showed that the animals of T6 group had a significant increase of 5% in the dentin apposition rate when compared with the control animals (C6). In the T6 group, ALP plasma levels were 25% higher than those in the C6 group. The results obtained from the knoop microhardness testing, demonstrated that the animals of T10 group showed greater microhardness than did the control animals (C10) (10%). In addition, EDX microanalysis showed that the P (23%) and Ca (53%) atom % content in the peritubular dentin was increased in the T10 group, compared with C10 group. The Ca/P ratio of T10 animals was also higher than the C10 animals

(24%). The chemical composition of intertubular dentin did not differ between the groups. These results indicate that the short-time PTH administration had an anabolic effect on the dentin formation of incisor teeth of young healthy mice; this effect was following by mechanical and composition changes in the dentin.

Key words: parathyroid hormone, dentin, knoop microhardness, EDX, mice.

SUMÁRIO

INTRODUÇÃO

O hormônio paratireóideo (PTH) é um polipeptídeo de 84 aminoácidos sintetizado pelas quatro glândulas paratireóides (Swarthout *et al*., 2002), sendo que o fragmento PTH (1-34) constitui os primeiros 34 aminoácidos da cadeia polipeptídica do hormônio e produz os seus principais efeitos biológicos (Neer *et al*., 2001).

Desde o início do século XX, o PTH vem sendo estudado, mas somente a partir da década de 70, após a bem sucedida síntese química do PTH humano (1-34), é que ele pôde ser investigado com mais detalhes (Frolik *et al*., 2003).

Por ser o principal regulador da homeostasia dos íons minerais, cálcio e fosfato, nos rins e ossos (Habener *et al.,* 1984), e indiretamente no intestino (Fleet *et al*., 1994), o PTH funciona como um mediador na remodelação óssea (Strewler *et al.,* 1987), o que é fisiologicamente observado na sua ação em mobilizar estes íons, quando necessário, pela estimulação da atividade osteoclástica (Neer *et al*., 2001; Horwitz *et al*., 2003; Frolik *et al*., 2003).

O PTH estimula a formação e a reabsorção óssea, e pode aumentar ou diminuir a massa óssea dependendo da sua forma de administração (Neer *et al*., 2001; Horwitz *et al*., 2003). A administração intermitente de PTH tem um efeito anabólico no tecido ósseo, podendo tratar os efeitos da osteoporose, o que já foi demonstrado em trabalhos com humanos e roedores (Hodsman *et al.,* 2005), sendo atualmente a única droga anabólica óssea permitida para uso em humanos (Food And Drug Administration - FDA, 2000). A administração intermitente de PTH também é um efetivo tratamento para osteoporose induzida por glucocorticóides (Saag *et al*., 2009), uma condição que, assim como o envelhecimento, é caracterizada por uma diminuição da formação óssea. Adicionalmente, foi demonstrado que o efeito anabólico do PTH é capaz de acelerar o reparo de fraturas ósseas em macacos (Manabe *et al.,* 2007), e em ratos (Warden *et al.,* 2009).

Embora o completo mecanismo que gera este anabolismo não seja totalmente compreendido, sabe-se que o aumento da massa óssea provocada pela administração intermitente de PTH, pode estar relacionado a uma estimulação da proliferação e diferenciação de osteoblastos, bem como, uma diminuição da taxa de apoptose destas células (Swarthout *et al*., 2002; Jilka, 2007). Esses efeitos são obtidos pela exposição repetida e transitória das células ósseas ao PTH, já que o hormônio é eliminado da circulação dentro de 2-3 horas após a administração (Lindsay *et al.,* 1993; Frolik *et al.,* 2003; Bellido *et al.,* 2005).

Muitos trabalhos mostram um papel crítico da via de sinalização Wnt no efeito anabólico induzido pelo PTH (Bodine *et al*., 2007; Kramer *et al*., 2010a, Kramer *et al*., 2010b). Mais especificamente, o PTH estimula a via de sinalização Wnt ⁄ b-catenina, ativando a LRP6 (Wan *et al*., 2008) e inibindo a síntese de esclerostina e outros antagonistas da Wnt (Bellido *et al*., 2005; Keller & Kneissel, 2005; Bodine *et al*., 2007; Guo *et al*., 2010).

Além disso, este hormônio também é capaz de modular a expressão gênica de proteínas da matriz extracelular (Locklin *et al.,* 2003; Turner *et al*., 2007), levando a um aumento na deposição de matriz em vários ossos, inclusive na mandíbula (Miller *et al*., 1997; Manabe *et al.,* 2007; Jilka, 2007).

Embora, a maioria dos estudos encontrados na literatura que investigaram as funções do PTH, envolve células ósseas, outros tipos celulares também podem responder ao estímulo por este hormônio. Dentre as células não ósseas que podem responder a exposição ao PTH, podemos citar as células do ligamento periodontal (Lossdörfer *et al*., 2005; Lossdörfer *et al*., 2006) as células da polpa dentária (Nagata *et al.,* 1989; Hamasaki *et al*., 1992) e odontoblastos (Lundgren *et al*., 1998; Calvi *et al*., 2004; Kato *et al*., 2005).

A dentina é o mais volumoso tecido mineralizado do dente, sendo recoberta pelo esmalte na porção coronária e pelo cemento na porção radicular. Este complexo tecido de natureza conjuntiva é produzido por células denominadas odontoblastos, constituído por uma porção orgânica (proteínas colágenas e não-colágenas) e por outra porção mineralizada (hidroxiapatita). A maior parte da

dentina é composta de dentina intertubular e peritubular, permeada pela matriz contendo os túbulos dentinários com os processos odontoblásticos (Linde & Goldberg, 1993; Butler *et al*., 2003).

As dentinas, peritubular e intertubular, têm diferentes propriedades mecânicas que revelam a distinta composição ultraestrutural e bioquímica, assim como o mecanismo de mineralização (Gotliv & Veis, 2007; Habelitz *et al*., 2007). Enquanto a dentina intertubular tem uma matriz orgânica colágena, a dentina peritubular possui uma especializada matriz não-colagenosa, rica em fosfoproteínas e Gla-proteínas secretadas pelas odontoblastos. Tanto as fosfoproteínas quanto as Gla-proteínas têm uma elevada afinidade pelos íons cálcio e pode induzir a nucleação da apatita, sugerindo um papel indutor na mineralização da parede tubular para um grau mais elevado do que a dentina intertubular (Gotliv & Veis, 2007; Habelitz *et al*., 2007).

Similaridades entre osso e dentina, no que diz respeito a natureza da matriz extracelular, sugerem que os mecanismos de osteogênese e dentinogênese se assemelham, especialmente no processo de biomineralização. Durante a formação do osso e dentina, osteoblastos e odontoblastos secretam uma matriz não mineralizada, rica em colágeno, denominadas osteóide e pré-dentina, respectivamente (Qin *et al*., 2002; Butler *et al*., 2003; MacDougall *et al*., 2006).

Entretanto, outras características evidenciam variações e especificidade nestes dois processos, em particular no que diz respeito aos níveis de proteínas da matriz extracelular (Butler *et al*., 2003; MacDougall *et al*., 2006). Outra diferença entre dentina e osso é que a dentina não participa da homeostase do cálcio do organismo. Em contraste com osso, a dentina, normalmente não é remodelada, assim nenhum processo de reabsorção ocorre normalmente neste tecido (Veis *et al*., 1993; Linde & Goldberg, 1993).

O entendimento dos processos de formação e biomineralização da dentina e a busca por novas terapias tornam-se importantes ao passo que, em alguns casos, onde há perda ou danos ao tecido dentinário e pulpar, tais como, cáries, processos restaurativos, atritos ou outros tipos de traumas, há a necessidade da

formação de uma nova dentina, denominada dentina terciária (Linde & Goldberg, 1993). Dependendo do tipo e da extensão da injúria, a dentina terciária é classificada em dois subtipos, dentina reacional e dentina reparativa. A dentina reacional é produzida por odontoblastos pré-existentes e contém túbulos que são contínuos com a dentina secundária. Já a dentina reparativa, é produzida por células progenitoras da polpa que se diferenciam em odontoblastos e exibe, primeiramente, um aspecto osteóide (Aguiar & Arana-Chavez, 2010).

Os dentes incisivos de roedores crescem continuamente durante a vida do animal, fazendo deles um importante modelo para o estudo do processo de formação de dentina. No incisivo de camundongos, a dentina da região subjacente ao primeiro molar é rapidamente mineralizada, e está, portanto, em um estágio ideal para medição da taxa de aposição dentinária (DenBesten *et al*., 2001).

Muitas pesquisas têm demonstrado a expressão e contribuição do PTHrp (peptídeo relacionado ao PTH) e/ou PTHR1 (receptor de PTH do tipo 1) no desenvolvimento de dentes humanos e de roedores (Beck *et al*., 1995; Lee *et al*., 1995; Tenorio & Hughes, 1996; Liu *et al*., 1998; Lundgren *et al*., 1998; Philbrick *et al*., 1998; Kitahara *et al*., 2002; Wysolmersky *et al*., 2001; Comier *et al*., 2003; Calvi *et al*., 2004).

Embora os efeitos do PTH na formação de dentina já tenham sido discutidos por trabalhos na literatura (Yonaga, 1978; Turnbull *et al*., 1983; Chardin *et al*., 1995; Miller *et al*., 1997), os resultados destes estudos não são conclusivos quanto aos efeitos e funções que este hormônio desempenha na dentinogênese. Turnbull *et al*. (1983) mostraram que, dependendo da dose, o PTH estimula a aposição de dentina em ratos paratireoidectomizados, já Miller *et al*. (1997) demonstraram que a administração intermitente de PTH não teve efeito na formação de dentina em ratas velhas ovarectomizadas.

Recentemente, em um trabalho que resultou em tese de doutorado de um dos alunos do nosso laboratório (Vasconcelos, 2008), foi observado que a administração intermitente de PTH pode acelerar o processo de reparo periodontal em ratos, além de aumentar a área de cemento neoformado após o reparo. Isso

foi verificado em um modelo em que é feita a remoção mecânica da porção média do periodonto de inserção na face vestibular da raiz mesial do primeiro molar inferior. Durante as análises histológicas, em que se verificou o efeito da administração intermitente de PTH no tipo de reparo descrito, foi observado que a raspagem do cemento dentário gerava a formação de uma dentina reacional referente à região instrumentada. Apesar de não ter sido feita uma análise histométrica, foi observado nos cortes histológicos que, aparentemente, havia uma maior porção de dentina reacional nos animais tratados com PTH. Tal achado foi o principal motivador para o delineamento do presente estudo.

Sendo assim, este trabalho teve como objetivo investigar o efeito da administração intermitente de hPTH (1-34) na taxa de aposição de dentina e nas características estruturais desta dentina formada em incisivos de camundongos jovens e saudáveis.

CAPÍTULO 1

Effect of parathyroid hormone administration on dentin formation in mice

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Abstract

Parathyroid hormone (PTH) is an important factor for mineralized tissues; it controls matrix deposition and biomineralization. However, the role of this hormone on dentin formation is poorly known. The purpose of this study is to investigate the effects of intermittent PTH administration on the apposition rate and structural features of dentin from mouse incisors. Young male A/J Unib mice were treated daily for 6 and 10 days with 40µg/Kg of hPTH (1-34) or a vehicle. Dentin apposition rates, measured by fluorescent labels (tetracycline and calcein), and alkaline phosphatase (ALP) plasma levels were evaluated after 6 days of treatment. Knoop microhardness testing and element content measurements in atom % of calcium (Ca), phosphorus (P), oxygen (O), and magnesium (Mg) in the peritubular and intertubular dentin were performed by Energy Dispersive X-ray (EDX) microanalysis via Scanning Electron Microscopy (SEM) after 10 days of treatment. Histometric analysis revealed an increase of 5% in the apposition rate of dentin and 25% in the ALP plasma levels in the PTH treated group. In addition, knoop microhardness testing revealed that the animals treated with PTH had a greater microhardness (11%). EDX microanalysis showed that PTH treatment led to increases in P (23%) and Ca (53%) atom % content, as well as the Ca/P ratio (24%) in peritubular dentin. The chemical composition of intertubular dentin did not vary between the groups. These findings indicate that intermittent administration of hPTH(1-34) has an anabolic effect on the dentin formation in incisors of young mice.

Keywords: *parathyroid hormone, dentin, knoop microhardness, EDX, mice.*

Introduction

After the successful chemical synthesis of human PTH in the 1970s, numerous studies have investigated the effects of PTH administration both *in vivo* in detail [1-2] and *in vitro* [3-4]. It is known that PTH may stimulate bone formation and resorption, and may increase or decrease bone mass, depending upon the method of administration [5]. The effects of PTH on bone metabolism are mediated by its binding to G-protein-coupled receptors (GPCR) on stromal and osteoblastic cells [2-3].

The primary role of PTH, an 84-amino acid peptide that is produced by the parathyroid gland, is related to calcium homeostasis. PTH directly increases renal tubular calcium reabsorption and indirectly enhances intestinal calcium absorption. The normal physiological role of PTH on skeletal homeostasis, when secreted endogenously, is more complex, and its main function is to regulate bone remodeling rather than overall skeletal mass [2].

Intermittent PTH administration has an anabolic effect, increasing bone formation over resorption, resulting in increased bone mass. Thus, human parathyroid hormone (hPTH 1–84) and its analog, recombinant hPTH 1–34, can treat osteoporosis, which was demonstrated in studies with rodents [1, 6] and humans [2, 5]. Additionally, it was shown that the anabolic effect of PTH is able to accelerate the repair of bone fractures in monkeys [7] and rats [8].

Although many cell types, such as periodontal ligament cells [4], dental pulp cells [9-10], and odontoblasts [11-13], can respond to PTH, most studies that investigated the effects of this hormone used bone cells. Furthermore, PTH-related peptide (PTHrp), a peptide with similar biological activity as that of PTH, is known to play an important role in tooth development because the deletion of the PTHrp-gene impairs tooth eruption, resulting in distortion of the anatomy of the developing tooth [14].

Dentin, the most voluminous mineralized tissue of the tooth, is formed by odontoblasts in a process called dentinogenesis. Similarities in the overall nature of the bone and dentin extracellular matrix (ECM) proteins and the fact that each tissue is first synthesized as an unmineralized collagen-rich matrix (i.e., osteoid and predentin) strongly suggest that the mechanisms of osteogenesis and dentinogenesis, specially in the

mineralization process, resemble each other in critical steps [15-17]. However, compared with the study of osteogenesis, dentinogenesis has several advantages as a model for experimental studies of biomineralization mechanisms [18-19].

Despite this likeness, other features bespeak variations and specificity in these two processes, particularly with regard to the levels of ECM proteins [16-17]. Another difference between dentin and bone is that dentin does not participate in the calcium homeostasis of the organism. In contrast to bone, dentin is normally not remodeled; no resorptive processes normally occur in the tissue [18-19].

Because the functions of the PTH and treatment effects of this hormone in dentin formation is poorly known, this study was designed to determine whether intermittent PTH administration could affect the formation and structural features of dentin in young mice incisors.

Materials and Methods

Animals

Forty male A/J Unib mice (8-weeks old, starting weight: approximately 22g) obtained at the Animal Facility Center of the University of Campinas, were maintained in a room with 12 hour day/night cycles with food and drinking water ad-libitum. Experimental procedures were approved by the Institutional Animal Research Committee at the University of Campinas (São Paulo, Brazil).

Experimental Period

The animals were randomly assigned into two groups: Twenty animals received daily subcutaneous injections of 40µg/Kg of hPTH (1-34) (Sigma-Aldrich, St. Louis, MO, USA), diluted in 0.01% acetic acid. The remaining twenty animals received the vehicle (0.01% acetic acid) under an identical protocol, which served as control group. Each group was divided further into two groups, which were treated during 6 days and 10 days, respectively. Therefore, this resulted in four subgroups for analysis (10 animals/group): (T6) PTH-treated per 6 days; (T10) PTH-treated per 10 days; (C6) vehicle-treated per 6

days; (C10) vehicle-treated per 10 days (figure 1). The intermittent PTH-dose and vehicle used in the present study were based on previous studies [20-21]. The animals of C6 and T6 groups received intraperitoneal injections of fluorescent markers twenty-four hours prior to the start of treatments (tetracycline, Sigma-Aldrich, USA, 15mg/kg), and on the last day of treatment (calcein, Sigma-Aldrich, USA, 15mg/kg) (figure 1).

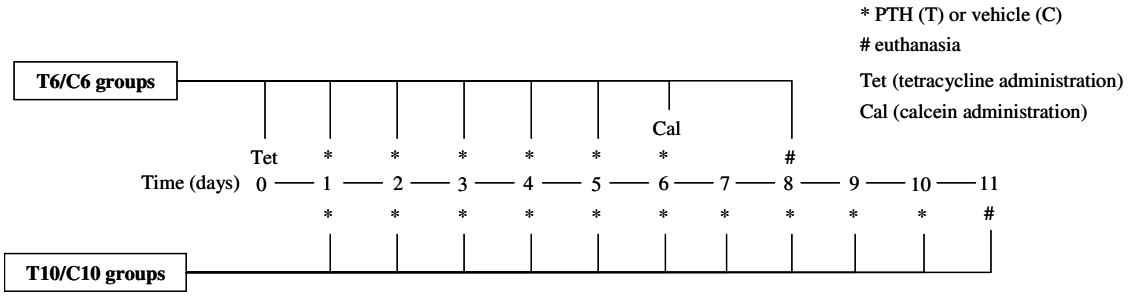

Figure 1 – Schedule of experimental period. Eight-week-old animals were treated with PTH (hPTH 1-34, 40µg/kg) or vehicle (acetic acid 0,01%) per 6 and 10 days. Both fluorescent markers, tetracycline and calcein (15mg/kg), were administered in the animals of the T6 and C6 groups (see material and methods section).

Tissue Preparation

C6 and T6 groups: Two days after calcein administration, the animals were anesthetized with ketamine (100mg/Kg, Vetbrands Brasil Ltda., SP, Brazil) and euthanized by puncture of the left heart ventricle, and blood samples were taken in plastic tubes that had been previously prepared with heparin (5000UI/ml, Hipolabor Farmacêutica Ltda., MG, Brazil), immediately centrifuged at 4000rpm for 5 minutes, and the supernatant plasma was stored at -70°C to detect alkaline phosphatase (ALP) levels. The left hemimandibles were removed and fixed in 4% formaldehyde solution (Dinâmica®, SP, Brazil) for 48 hours for analysis of the dentin apposition rate.

C10 and T10 groups: After 10 days of treatment with PTH or vehicle, the animals were anesthetized with ketamine and euthanized by cervical dislocation; the left and right hemimandibles were removed and frozen at -20°C for later knoop microhardness testing

and Energy Dispersive X-ray (EDX) microanalysis by Scanning Electron Microcopy (SEM).

Dentin Apposition Rate

After fixation, the left hemimandibles from C6 and T6 groups were dissected, dehydrated, and embedded undecalcified in polymethyl methacrylate (PMMA) (VIPI FLASH, SP, Brazil). Cross-sections of the hemimandible at the first molar region, obtained by low speed saw (Model 650) (South Bay Technology, CA, USA), were wet-polished to a final thickness of 80µm (Figure 2a, b). The slices were observed using a fluorescence microscope (Leica DM LP) (Leica Microsystems Inc., Wetzlar, Germany) and measurements of the distance between two fluorescent labels at 8 geometrically equal intervals around the incisor were performed (Figure 2c) using a image analysis software (Image-Pro® Plus 4.5) (Media Cybernetics, Inc., MD, USA). The mean of all the measurements (measured by one blinded, previously calibrated examiner) was expressed as the total apposition for each animal, and then divided by the time between administrations of the fluorescents markers to give the dentin apposition rate per day. It is important to comment here, that this study was reproduced twice under the same conditions.

Alkaline Phosphatase (ALP) Plasma Levels

ALP plasma levels from C6 and T6 groups were measured as the release of thymolphthalein from thymolphthalein monophosphate using a commercial kit (Labtest Diagnostica S/A, MG, Brazil). Briefly, 50µl of thymolphthalein monophosphate were mixed with 0.5ml of diethanolamine buffer, 0.3mmol/ml (pH=10.1), and left for 2 minutes at 37°C. Afterwards, 50µl of the plasma sample was added. This stood for 10 minutes at 37°C, then 2ml of a solution of Na_2CO_3 (0.09 mmol/ml) and NaOH (0.25 mmol/ml) was added to allow color development. The absorbance was measured at 590nm by ELISA (Molecular Devices, CA, USA), and ALP levels were calculated from a standard solution and data are expressed as U/L of ALP.

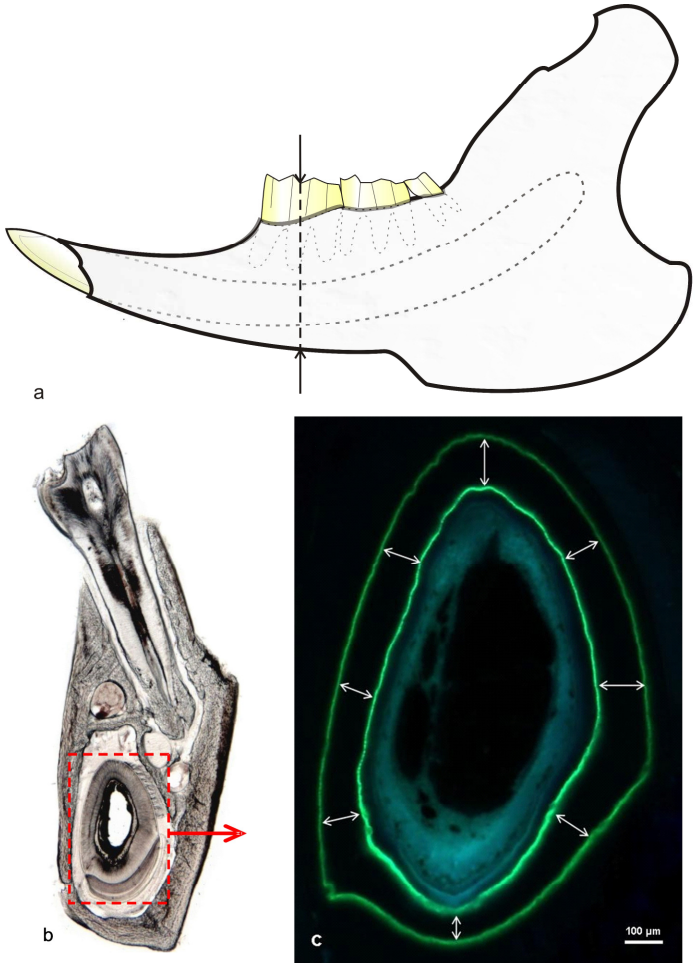

Figure 2 – **(a)** Illustration pointing out the hemimandible region where cross-sections were performed (black dashed line) for dentin apposition rate analysis; **(b)** Photomicrography of an undecalcified cross-section of the first molar region focusing on the lower incisor (red arrow) (magnification level, x25); **(c)** Image of a lower incisor, obtained via fluorescent microscopy, showing the regions where the distance between two fluorescent labels were measured (external label = tetracycline; internal label = calcein) (white arrows) (magnification level, x100; bar = 100µm).

Knoop Microhardness Testing

The left hemimandibles from C10 and T10 groups were sectioned transversely at the first molar region (Figure 2a, b). The fragments obtained were embedded in epoxy resin

(Buehler, Lake Bluff, IL, USA) and wet-polished for microhardness testing (Future Tech - FM-1E, Tokyo, Japan). Five indentations were performed at dentin mesial face of incisor, each separated 200µm from another. Indentations were done with a 25g load during 5 sec. The mean of the values obtained from indentations were expressed as Knoop Number Hardness (KNH).

EDX Microanalysis

Initially, it was observed in a pilot study that after polishing the incisor mesial face at a 200µm depth into the tooth (from the outer surface), the dentin tubules were arranged transversely, exposing the peritubular and intertubular dentin features (Figure 3). For the EDX microanalysis, the right hemimandibles of C10 and T10 were cross-sectioned at the first molar region (Figure 3a). The incisors were extracted and the mesial face was wet-polished at a 200µm depth from the outer surface, using 1200 and 2000-grit silicon carbide papers (Norton S/A, SP, Brazil) (Figure 3b). After ultrasonic cleaning in deionized water (B- 1210-MTH, Branson Ultrasonic Corporation, CT, USA), the specimens were dehydrated in alcohol until 100% and then dried in a recipient containing silica gel. The specimens were carbon-coated (Desk II Sputtering, Denton Vacuum, NJ, USA) and the elemental content of dentin was analyzed using EDX microanalysis by images obtained in SEM (JXA-840A; JEOL, Tokyo, Japan) under 25kV and x3000 magnification. For each specimen, six images were obtained (1200µm^2 each) (Figure 3c,d) and the element content in atom% of calcium (Ca), phosphorus (P), oxygen (O) and magnesium (Mg) were measured in the peritubular and intertubular dentin; later, the Ca/P ratio was calculated.

Statistical Analysis

All results are expressed as mean ± standard deviation (SD). Statistical analysis of all tests was completed using unpaired Student's t test (two-tailed). Statistical significance limit was set at 5%, ($p<0.05$).

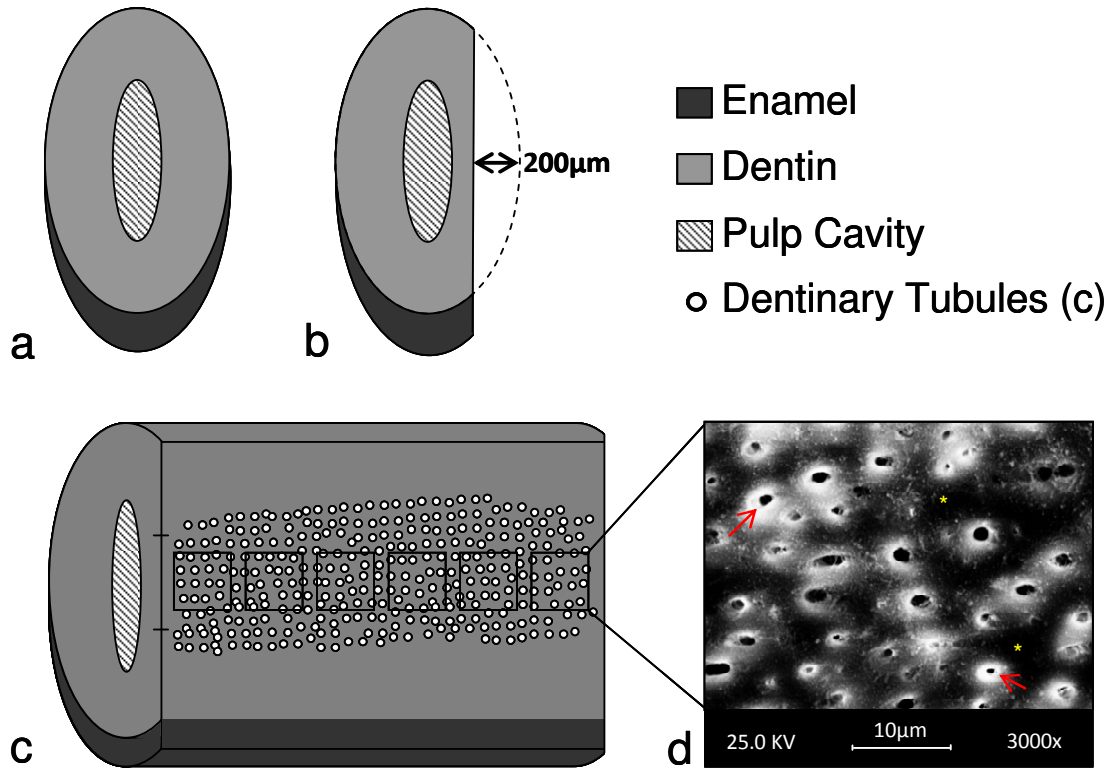

Figure 3 – Schematic drawing demonstrating the incisor preparation for EDX microanalysis in SEM. **(a)** Cross-section of incisor; **(b)** Incisor mesial face polished at a 200µm depth from the outer surface; **(c)** Longitudinal view of the incisor after polishing, indicating that the EDX microanalysis was performed at the middle third of the dentin mesial face (black squares); **(d)** SEM image of a carbon-coated specimen showing the transversely-arranged dentin tubules, especially the peritubular dentin (red arrows) and intertubular dentin (yellow asterisk) features (Accelerating voltage = 25 KV and Magnification = x3000).

Results

Histometrical analysis of the cross-sections in fluorecence microscopy showed that the animals submitted to intermittent administration of PTH (T6) presented a significant increase of 5% in the dentin apposition rate when compared with the control animals (C6). As described in the Material and Methods section, this experiment was replicated twice

under the same conditions and the histometrics findings were similar. Here, we showed only the data of one of two sets of the experiments conducted for analysis of dentin apposition rate (table 1). In the T6 group, ALP plasma levels were 25% higher than those in the C6 group (table 1).

Table 1 – Dentin apposition rates and plasma levels of alkaline phosphatase (ALP) in mice submitted to intermittent administration of parathyroid hormone (T6) and placebo (C6 = Control). Data are expressed as mean ± standard deviation (SD).

Groups	**n**	**Dentin apposition rate (µm/day)**	**ALP (U/L)**
C6	10	18.07±0.95	41.87±7.59
T6	10	*19.05±1.03	*52.33±4.00

* Significantly different from Control group (C6) to student's *t* test, $p < 0.05$.

The results obtained from the knoop microhardness testing, performed on the mesial face of dentin cross-sectioned incisors, demonstrated that the animals that were treated daily with PTH over 10 days (T10) showed greater microhardness than did the control animals (C10) (10%), as shown in Figure 4.

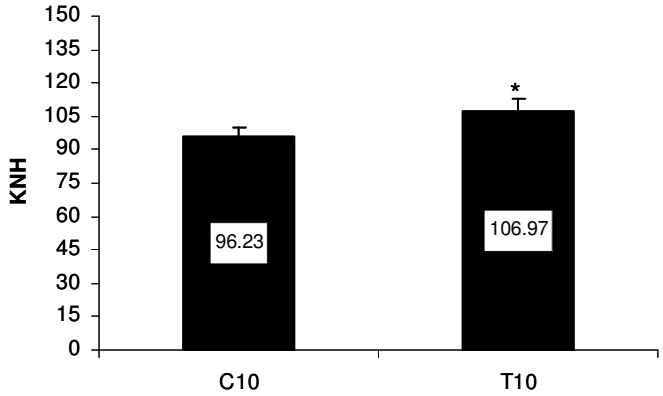

Figure 4 – Dentin knoop number microhardness (KNH) means and standard deviations from animals treated daily with PTH per 10 days (T10) and placebo (C10 = Control).
* Significantly different than control group to student's *t* test ($p < 0.01$).

To evaluate changes in the chemical composition of dentin submitted to PTH treatment, the elemental contents of peritubular and intertubular dentin were measured by EDX microanalysis (Table 2). The P (23%) and Ca (53%) atom % content in peritubular dentin was increased in the T10 group, compared with C10 group. The Ca/P ratio in peritubular dentin of T10 animals was also higher than the C10 animals (24%). The chemical composition of intertubular dentin did not differ between the groups.

Table 2 – Means ± standard deviations of element content (atom%) and Ca/P ratio of peritubular and intertubular dentin of mice submitted to intermittent administration of parathyroid hormone (T10) and placebo (C10 = Control).

Element	**Peritubular dentin**		**Intertubular dentin**	
	C10	*T10*	*C10*	*T10*
Mg atom%	1.24±0.4	0.89±0.2	1.13±0.3	1.04±0.3
O atom%	57.26±7.3	39.81±12.9	55.72±8.2	48.54±8.6
P atom%	18.83±2.4	*23.26±3.3	19.17±2.1	21.41±2.7
Ca atom%	23.49±4.6	*36.04±9.6	24.73±5.9	29.00±5.9
Ca/P ratio	1.234±0.1	*1.525±0.2	1.273±0.1	1.342±0.1

* Significantly different from control group to student's *t* test ($p < 0.01$)

Discussion

Dentinogenesis is a continuous process of matrix deposition during the life of a tooth. Rodent incisors grow continuously throughout the animal's life, making them an important model of the dentin formation process [13, 22]. In mice, dentin from the incisor region underlying the first molar is rapidly mineralized, and is therefore at an ideal stage for the measurement of the mineral apposition rate. Both tetracycline and calcein bind to newly formed mineral, which then fluoresces under UV light [23]. In the present study, it was demonstrated, by measurements using fluorescents markers, that the hPTH(1-34) causes an anabolic effect on dentin deposition during incisor formation in young mice.

The molecular mechanisms that underlie the formation of the mineralized tissues have not been fully elucidated, and it is known that PTH is an important hormone in

controlling matrix deposition and biomineralization. Although the effects of PTH on dentin formation have been discussed [1, 24-26], this study is a first report to investigate the intermittent hPTH(1-34) administration effect on the quality and appositional rate of dentin during incisor formation in healthy young mice.

The anabolic effect of PTH has been well reported by studies involving bone tissue, and bone anabolism can be detectable after 10 days of PTH treatment [27]; these effects consisted of an increase in bone mass and mineral content. Here, short-term PTH administration results in an increase of the ALP blood levels. The analysis showed that in T6, the ALP blood level was significantly higher than it was in C6 (25%) (Table 1). ALP is a marker of bone turnover [28] and changes in the release of ALP may indicate an action of PTH intermittent administration [29].

Lundgren et al. [11] showed an anabolic response of odontoblasts for PTH (1-34) treatment, and verified that this response is mediated through the protein kinase A/cAMP pathway. Some studies reported that PTHrp and PTH (1-34) share a common receptor: type 1 PTH/PTHrp receptor (PTHR1) [30-31], which is also expressed in odontoblasts [13]. Calvi et al. [12] showed that, in the tooth development, odontoblastic expression of the activated PTHR1 resulted in decreased dentin in the molar crowns, whereas the incisors had large amounts of dentin. These data therefore, suggest that in odontoblasts, activation of the PTHR1 triggers responses similar to those in osteoblasts, with expansion of the odontoblastic pool and changes in odontoblastic maturation and function

PTH acts directly in the renal tubular to increase calcium resorption [32], and indirectly to enhance intestinal calcium absorption [33]. Although the anabolic effects of PTH on bone tissue has been linked to increased cell proliferation and differentiation, and the attenuation of cell apoptosis [3, 34], some reports have shown that this hormone is also able to modulate gene expression of matrix components [35-36], leading to an increase in the apposition of matrix in several bones, including mandible, [1, 7, 34].

It is important to know how the tooth quality, which relates to the ability of the tooth to fulfill its functions, is affected by PTH intermittent administration. Tooth quality can be analyzed by measuring tooth material and mechanical properties [37]. Material properties are those properties specific (intrinsic) to a material, whereas mechanical

properties are those properties that reveal the reaction, either elastic or plastic, of a material to an applied stress [37]. In this study, material properties were analyzed by measuring elemental contents in the atom % of peritubular and intertubular dentin, calculating the Ca/P ratio, whereas mechanical property was analyzed using the degree of mineralization of dentin.

EDX microanalysis, used for measuring the element content in atom% of calcium (Ca), phosphorus (P), and the Ca/P ratio in the peritubular and intertubular dentin in this study, indicated important changes in the composition of apatite from dentine following PTH treatment (Table 2). For the peritubular dentin, the P (23%) and Ca (53%) atom% content was increased in T10 animals when compared to C10 animals. In addition, the Ca/P ratio in the peritubular dentin of T10 animals was higher than C10 animals (24%), which not was observed in the intertubular dentin.

The peritubular and intertubular dentin have different mechanical properties that reveal the distinct ultrastructural and biochemical composition, such as the mineralization mechanism [38-39]. While the intertubular dentin has a collagen fibril-based matrix, the peritubular dentin is a specialized non-collagenous matrix that is rich in phosphoproteins and Gla-proteins secreted by the odontoblasts. Both phosphoproteins and Gla-proteins have a high affinity for calcium ions and can induce apatite nucleation, suggesting an inductive role in mineralization of the tubule wall to a higher degree than the intertubular dentin [38-39].

Different methods have been used to evaluate the degree of dentin mineralization and microhardness testing is the method of choice for detecting changes in the consistency of the surface, as mineral lost or gain [40-41]. The results obtained from the knoop microhardness testing, revealed that the animals of the T10 group presented a greater microhardness than did the control animals (C10) (10%), as shown in Figure 4. The higher elemental content in the peritubular dentin of the T10 animals, compared to that in the control animals, may have led to an increase in the microhardness of these animals, since there is a correlation between mineral content and the length of the indentations in microhardness testing [41].

The findings of this study demonstrated that, in mice, PTH can to generate more calcified dentin compared to regular dentin. This response to the hormone could be further investigated as a potential therapy in diseases that commonly affect the dentin formation as X-linked hypophosphatemic rickets (XHLR) or dentinogenesis imperfecta. In XHLR for example, is common found dentinal defects characterized by hypocalcified and interglobular dentin in both dentitions, enlarged pulp chambers with pulp horns extending to the dentin-enamel junction, and spontaneous dental abscesses without caries or history of trauma. In this case, some therapy that can increase dentin formation and mineralization will be very interesting [42].

In summary, the results showed an anabolic effect of short-term PTH administration in the dentin formation of incisor teeth of young healthy mice; this effect was following by mechanical and compositional changes in dentine. Furthermore, other investigations are need attempting to understanding cellular and molecular mechanisms of PTH action on dentin formation.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

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CONCLUSÃO

A administração intermitente de PTH teve um efeito anabólico na formação de dentina em camundongos jovens e saudáveis. Este efeito foi acompanhado de importantes alterações estruturais, levando a formação de uma dentina com maior grau de mineralização.

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ANEXO 1

Calcified Tissue International

Effect of Parathyroid Hormone Administration on Dentin Formation in Mice.

Journal:	*Calcified Tissue International*
Manuscript ID:	CTI-10-0217.R1
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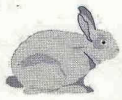
Keywords:	Peptide Hormones: PTH/PTHrP, Dental Matrix Biology, Odontoblasts, Ameloblasts, Cementoblasts

ANEXO 2

UNICAMP



CEEA/Unicamp

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

C E R T I F I C A D O

Certificamos que o Protocolo nº **1762-1**, sobre **"Avaliação do efeito do tratamento intermitente com hormônio paratireóideo (PHT) no tecido dentinário em camundongos"**, sob a responsabilidade de **Prof. Dr. Marcelo Rocha Marques / Gustavo Narvaes Guimarães**, 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/Unicamp em **09 de fevereiro de 2009**.

C E R T I F I C A T E

We certify that the protocol nº **1762-1**, entitled **"Effect of the intermittent parathyroid hormone (PHT) administration on dentin tissue in mice"**, 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 **February 9, 2009**.

Campinas, 09 de fevereiro de 2009.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

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