MÔNICA GRAZIELI CORRÊA

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FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Orientador: Prof. Dr. Enilson Antonio Sallum

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

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e orientada pelo Prof.Dr. Enilson Antonio Sallum.

Assinatura do orientador

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Dedico esse trabalho às pessoas mais importantes da minha vida:
minha mãe, Fátima, minha irmã, Diêniffer,
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Há duas formas para viver a sua vida:
Uma é acreditar que não existe milagre.
A outra é acreditar que todas as coisas são um milagre.

Albert Einstein

RESUMO

Fatores sistêmicos e comportamentais tem sido relacionados não somente com o início e progressão da doença periodontal, mas também com a resposta ao tratamento dessa doença. Os objetivos destes estudos foram avaliar o efeito das proteínas derivadas da matriz do esmalte (EMD) sobre reparo e regeneração de defeitos periodontais não contaminados em ratos submetidos a: i) diabetes mellitus induzida (DM); ii) estresse crônico (EC). **Métodos:** Para cada trabalho, foram designados os seguintes grupos: i) Vinte ratos Wistar foram aleatoriamente divididos em dois grupos: G1- Diabetes mellitus (DM) induzida com uma dose única intraperitoneal de estreptozotocina (STZ) (n=10); G2 – controle – não exposto a DM (n=10). Todos os animais foram submetidos à cirurgia de criação dos defeitos tipo fenestração bilaterais (4 mm de comprimento, 3 mm de altura e 1 mm de profundidade), com o objetivo de expor a região vestibular da raiz distal dos primeiros molares inferiores, e proporcionar remoção do cemento dental. Imediatamente após o procedimento cirúrgico, os defeitos de cada animal foram divididos aleatoriamente em 2 subgrupos: controle não tratado e tratado com EMD. Os animais foram sacrificados 21 dias após a criação dos defeitos e os seguintes parâmetros histométricos foram analisados: preenchimento do defeito (PD), densidade óssea (DO) e formação de novo cemento (FNC). O número de osteoclastos foi determinado histoquimicamente por meio da fosfatase alcalina resistente ao ácido tartárico (TRAP). Os defeitos de fenestração foram criados 7 dias após a indução do DM; ii) Vinte ratos Wistar foram aleatoriamente divididos em dois grupos: G1- estresse crônico (restrição de movimento e isolamento, 12h/dia) (n=10), G2= não exposto ao EC (n=10). Os defeitos de fenestração foram criados 15 dias após o início da indução do estresse. **Resultados:** i) os ratos submetidos ao diabetes mellitus (G1) apresentarm menor PD E DO, quando comparados com os ratos não expostos ao DM. EMD proporcionou maior PD em ambos os grupos (G1 e G2) e aumentou a DO e FNC somente em G2. O número de osteoclastos foi significativamente maior nos sítios tratados com EMD em G1, quando comparados com G2; ii) os ratos submetidos ao EC (G1) apresentaram menor DO, quando comparados com os ratos não expostos ao EC (G2). EMD aumentou o PD somente em G1 e aumentou a DO e FNC em ambos os grupos. O número de osteoclastos foi

significativamente maior em G1, quando comparado com G2, e nos sítios tratados com EMD de G1 e G2. **Conclusões:** Dentro dos limites desse estudo, pode-se concluir que: i) o diabetes mellitus pode gerar um efeito negativo sobre a densidade óssea. As proteínas derivadas da matriz do esmalte podem aumentar o preenchimento do defeito na presença do diabetes mellitus ou em condições normais. No entanto, EMD não aumenta significativamente a formação de novo cemento em animais diabéticos; ii) o estresse crônico produz um efeito negativo significativo sobre a densidade óssea. As proteínas derivadas da matriz do esmalte aumentam o preenchimento do defeito na presença do estresse crônico e aumentam a densidade óssea e formação de novo cemento na presença e na ausência do estresse crônico.

Palavras-chave: diabetes mellitus; estresse crônico; proteínas derivadas da matriz do esmalte; streptozotocina; ratos; regeneração.

ABSTRACT

Behavioral and systemic factors have been associated not only with the onset and progression of periodontal disease, but also with the outcomes of the treatment of this disease. The objectives of these studies were to evaluate the effect of enamel matrix derivative (EMD) treatment in tissue repair and regeneration in a periodontal fenestration model in rats subjected to: i) induced diabetes mellitus (DM); ii) chronic stress (CS). **Methods:** Bilateral fenestration defects (standardized with 4 mm in width, 3 mm in length and ≈ 1 mm deep) were created at the buccal aspect of the first mandibular molar of all animal. After the surgeries the defects of each animal were randomly assigned to two subgroups: non-treated control and treated with EMD. The animals were euthanized 21 days later and the percentage of defect fill, density of newly formed bone, and new cementum formation were histometrically assessed. The number of osteoclasts was determined by tartrate-resistant acid phosphatase. For each study, the following groups were established: i) Twenty Wistar rats were randomly assigned to two groups: G1- Diabetes mellitus was induced with a single intraperitoneal injection of streptozotocin (STZ) (n=10); G2= non-exposed to DM (n=10). Fenestration defects were created seven days after DM induction; ii) Twenty Wistar rats were randomly assigned to two groups: G1= CS – chronic stress (restraint stress for 12 hours/day) (n=10), G2= non-exposed to CS (n=10). Fenestration defects were created fifteen days after initiation of CS. **Results:** i) G1 (DM) showed less defect fill and bone density (BD) compared to G2. EMD provided an increased defect fill in both groups and enhanced BD and new cementum formation only in G2. The number of TRAP-positive osteoclasts was significantly higher in EMD-treated sites of G1; ii) G1 showed less bone density (BD) compared to G2. EMD provided an increased defect fill (DF) in G1 treated sites and higher BD and new cementum formation (NCF) in treated sites of both groups. The number of TRAP–positive osteoclasts was significantly higher in G1 when compared to G2 and in EMD-treated sites of both groups. **Conclusions:** Within the limits of these studies, it can be conclude that: i) DM may produce a significant detrimental effect on bone density. EMD may provide greater defect fill under diabetic or normal conditions; however, it may not significantly increase new

cementum formation in diabetic animals; ii) CS may produce a significant detrimental effect on bone density. EMD may provide greater defect fill in the presence of CS and increased bone density and cementum formation in the presence or absence of CS.

Key words: Diabetes mellitus; chronic stress; enamel matrix proteins; streptozocin; rats; regeneration.

PREFÁCIO

Esta tese está baseada nos seguintes artigos científicos:

1. Histometric analysis of the effect of enamel matrix derivative on the healing of periodontal defects in diabetic rats. *J Periodontol* 2012 Nov 3. [Epub ahead of print] (accepted).
2. Impact of chronic stress on the outcome of enamel matrix derivative treatment in rats: histometric analysis. *J Periodontol* (submitted).

SUMÁRIO

INTRODUÇÃO

O processo de cura de tecidos danificados requer a participação de moléculas inflamatórias e de células, envolvendo vários estágios: inflamação, formação de tecido de granulação e remodelação tecidual. O tipo de reparo depende da disponibilidade de células apropriadas, mediadores solúveis e produção de matriz extracelular (Aukhil, 2000). A matriz extracelular (MEC) executa um importante papel na regeneração periodontal, regulando os eventos necessários para a mesma. A síntese e a deposição de mediadores solúveis incluem factores de crescimento tais como o fator de crescimento derivado de plaquetas, fator de crescimento transformador β e fator de crescimento de fibroblastos, além de citocinas, incluindo a interleucina-1, fator de necrose tumoral e interleucina-4, linfocinas, como o interferon-g, bem como fibronectina e outras moléculas de adesão, acontecem coordenadamente à síntese e deposição de macromoléculas da MEC. Além disso, a MEC regula a expressão gênica de fatores de crescimento e seus receptores (Gumbiner, 1996; Kazlauskas, 2001; Walker et al., 2005; Berrier e Yamada, 2007).

A periodontite é uma doença de etiologia multifatorial, tendo como causa primária, a infecção causada pelas bactérias presentes no biofilme dental. Essa doença tem como sequela, a destruição tecidual causada (osso, ligamento periodontal e cemento) (Dentino et al., 2013). A cura dos tecidos periodontais pode ocorrer através do reparo (nova inserção, formação de epitélio juncional longo e adesão conjuntiva) ou através da regeneração periodontal, processo em que há a reconstituição funcional do osso alveolar, cemento e ligamento periodontal (Glossary of Periodontal Terms, 2001). Nos estágios iniciais, proteínas plasmáticas, principalmente o fibrinogênio, precipitam na região da lesão. Depois disso, um infiltrado inflamatório de neutrófilos descontamina a ferida, enquanto macrófagos migram para a região removendo células mortas e substâncias residuais, além de secretarem fatores de crescimento que estimulam a proliferação de fibroblastos, produção de matriz e angiogênese (Polimeni et al., 2006). Nos estágios de maturação e remodelação, o tipo de tecido formado dependerá, em grande parte, do fenótipo das células que povoarem a lesão (Melcher, 1976). Estudos têm demonstrado que células

mesenquimais indiferenciadas do ligamento periodontal são responsáveis, em parte, pela síntese do sistema de fibras dentogengivais, além de terem o potencial para diferenciar-se em cementoblastos e osteoblastos, sendo assim, essenciais para a regeneração periodontal (McCulloch e Melcher, 1983, Pitaru et al. 1994).

Fatores sistêmicos e comportamentais tem sido relacionados não somente com o início e progressão da doença periodontal, mas também com a resposta ao tratamento dessa doença (Nunn, 2003). Os fatores de risco aceitos e comprovados em estudos epidemiológicos e longitudinais são o tabagismo, o diabetes mellitus e associação de alguns tipos de microorganismos (Albandar, 2002). Existem também os indicadores ou potenciais fatores de risco que podem influenciar na prevalência e na severidade das periodontites e dentre estas condições, está o estresse (Genco, 1996).

O diabetes mellitus (DM) é um grupo de desordens metabólicas distintas por tolerância alterada à glicose ou alteração no metabolismo de carboidratos. Essas alterações são caracterizadas por defeito na secreção de insulina, por ação alterada de insulina ou ambas as situações, cujo resultado é a hiperglicemia. As formas mais comuns de DM incluem o tipo 1 (resultados de uma destruição celular auto-imune das células β do pâncreas) e tipo 2 (inclui os indivíduos que têm a resistência à insulina e, geralmente, têm deficiência relativa de insulina) (American Diabetes Association, 2010).

As alterações metabólicas que ocorrem no DM e que estão relacionadas a estados patológicos, como a síndrome metabólica, podem influenciar a capacidade de reparação dos tecidos periodontais. Estudos *in vivo* mostraram que o DM pode afetar a formação e remodelação do osso, e cicatrização de feridas em ratos (Goodman e Hori, 1984, Verhaeghe et a. 1990). Observou-se que a formação e deposição de matriz óssea foram diminuídas em animais diabéticos. Além disso, detectou-se um volume de osso reduzido através da redução da produção de osteoblastos, osteoclastos, osteóide e osteocalcina. Estudos em animais demonstraram o impacto negativo do DM sobre a retenção mecânica e osseointegração de implantes (Verhaeghe et a. 1990, Yama et al. 1997, Takeshita et al. 1998, Nevins et al. 1998, Giglio et al. 2000, MacCracken et al. 2000, Margonar et al. 2003).

Os mecanismos biológicos que têm sido estudados para validar e explicar a relação entre alterações teciduais DM e periodontal são: microangiopatia gengival, alterações microbianas, alterações na resposta imune do hospedeiro, diminuição da produção de colágeno e aumento da colagenase, defeito de quimiotaxia de leucócitos polimorfonucleares (PMN) (Taylor e Borgnakke, 2008), Manouchehr-Pour et al. 1981, King, 2008) e a metabolização de produtos finais de glicosilação avançada (AGEs) (American Diabetes Association, 2010, Schmidt et al. 1996). De acordo com Retzepi e Donus (2010), supressão da diferenciação, proliferação e /ou redução da capacidade de formação de osso por osteoblastos durante o período crítico de cicatrização precoce têm sido considerados como mecanismos patogenéticos plausíveis para a reduzida neoformação óssea no diabetes não controlado. Estas alterações podem diminuir a capacidade de cura e exacerbar a resposta inflamatória, o que leva a capacidade de reparação reduzida.

O estresse foi definido por Selye como uma resposta não específica do organismo a qualquer tipo de exigência. É um estado de resposta do organismo a forças que agem no corpo e quando superam a capacidade adaptativa levam a doenças de exaustão e, eventualmente, morte (Selye, 1976, McEWEN 2000).

Diante do agente estressor, o organismo responde com algumas mudanças fisiológicas decorrentes da ativação do eixo simpático do Sistema Nervoso Autônomo (SNA) ou da ativação do eixo Hipotálamo-Pituitário-Adrenal (HPA) (Dhabhar e McEwen, 1997). A resposta inicial ao estresse (reação aguda) ocorre, com duração de alguns minutos ou poucas horas, por meio da ativação do SNA estimulando a medula adrenal e secreção de catecolaminas como epinefrina e norepinefrina, o que aumenta a liberação de prostaglandinas e proteases (Genco et al., 1998). Já na resposta tardia ao estresse ou durante um período de estresse crônico, ocorre a ativação do eixo HPA que resulta na produção de hormônio liberador de corticotropina e de arginina vasopressina pelo hipotálamo (Ader et al., 1995). Esses hormônios estimularão a hipófise a produzir o hormônio adrenocorticotrófico (ACTH) que, por sua vez, agirá sobre o córtex da glândula adrenal, responsável pela produção de glicocorticóides (GC).

Nos humanos, o GC secretado é o cortisol e em ratos, a corticoesterona (Breivik et al. 2000). Os GC regulam uma série de funções corporais, incluindo efeitos supressivos através de mecanismos altamente específicos (Genco et al., 1998). O GC reduzem o número de linfócitos circulantes, monócitos e eosinófilos, além de inibir o acúmulo destas células nos sítios inflamatórios, *in vivo* (Cupps & Fauci, 1982). Ao nível molecular, os GCs agem sobre células inflamatórias incluindo macrófagos, neutrófilos, eosinófilos e mastócitos, inibindo importantes funções como quimiotaxia, secreção e degranulação (Schleimer et al. 1989). Além disso, os GCs suprimem a cascata da resposta imuno-inflamatória através da inibição da apresentação de antígeno feita pelo macrófago, inibição da proliferação de linfócitos e da diferenciação em células efetoras como linfócitos *helper*, linfócitos citotóxicos, células NK, e células B, formadoras de anticorpos (Snyder & Unanue, 1982). Os GC também possuem efeito inibidor sobre a produção de citocinas incluindo IL-1, IL-2, IL-3 e IL-6, fator de necrose tumoral (TNF-α), interferon-gama (IFN-γ) e mediadores inflamatórios derivados do ácido aracdônico como as prostaglandinas e leucotrienos (Schleimer et al. 1989).

Estudos em animais demonstraram maior perda óssea alveolar após a exposição dos animais ao estresse (Gaspersic et al. 2002, Nakajima et al 2006, Takada et al 2004, Semenoff Segundo et al 2010, Huang et al. 2011). Breivik et al. (2011) e Semenoff Segundo et al (2010) demonstraram que a hiperativação do eixo HPA parece desempenhar um papel importante no aumento da susceptibilidade à doença periodontal. Peruzzo et al. 2008, verificaram aumento de IL-1β/IL-1ra, IL-6/IL-10 e RANKL/OPG na presença do estresse crônico, favorecendo a destruição óssea periodontal. Estudos clínicos (Moss et al. 1996, Genco et al. 1999, Hugonson et al. 2002, Hilgert et al. 2006) estabeleceram que a hiperativação do eixo HPA pode aumentar o risco para doença periodontal. Gameiro et al. (2008) investigaram o efeito do estresse sistêmico sobre as reações biológicas que ocorrem durante a movimentação ortodôntica. Eles verificaram que o estresse crônico ocasionou aumento da reabsorção óssea e do número de osteoclastos durante o movimento ortodôntico. Vandevska-Radunovic & Murison (2010) observaram que ratos sob estresse emocional tiveram menor quantidade de cemento celular. Além disso, o estresse

psicológico e o hipercortisolismo tem sido relacionados a efeitos diretos sobre o metabolismo ósseo, tais como redução da densidade mineral óssea (Chiodini et al. 2008) e inibição da atividade de osteoblastos (Ogoshi et al. 2008, De Nijs, 2008). O estímulo fisiológico do sistema nervoso simpático pode modular a atividade metabólica do osso, detectada pela expressão de mRNA de IL-6 em calvária de ratos (Kondo e Togari, 2003).

As proteínas derivadas da matriz do esmalte (EMD) tem sido utilizadas em procedimentos periodontais regenerativos, devido ao seu papel fundamental no desenvolvimento do cemento (Hammarstrom et al. 1997). Uma série de estudos *in vitro* demonstrou que EMD possui a capacidade de estimular a produção de osteoblastos e inibir a osteoclastogênese, favorecendo assim a neoformação óssea (He et al. 2004, 2005, Pischon et al, 2006). Estudos *in vivo* indicam que EMD é uma alternativa para regeneração periodontal em defeitos de furca e intra-ósseos (Tonetti et al. 2002, Donos et al. 2003, Sculean et al, 2005). Além disso, estudos clínicos longitudinais e controlados com defeitos intra-ósseos, demonstraram ganho de inserção e redução de profundidade de sondagem significativos com o uso de EMD (Pontoriero et al. 1999, Tonetti et al. 2002, Sculean et al. 2001, 2005) e estudos com lesões de bifurcação apresentaram resultados favoráveis quando utilizado o EMD em lesões de bifurcação classe II mandibulares (Donos et al. 2003, Jepsen et al. 2004, Meyle et al. 2004, Hoffman et al, 2006, Casarin et al. 2008).

Os achados descritos sugerem a possibilidade de redução da capacidade de cicatrização ou reparo dos tecidos periodontais sob a influência dos referidos fatores sistêmicos e comportamentais. No entanto, existe uma falta de informação, a partir de estudos *in vivo*, sobre o possível efeito desses fatores sistêmicos e comportamentais na capacidade de cicatrização de tecidos periodontais (ligamento periodontal, osso e cemento), após tratamentos regenerativos. Diante do exposto, os estudos tiveram como objetivos avaliar o efeito das proteínas derivadas da matriz do esmalte sobre reparo e regeneração de defeitos periodontais não contaminados em ratos submetidos a: i) diabetes mellitus induzida (DM); ii) estresse crônico (EC).

CAPÍTULO 1

Histometric analysis of the effect of enamel matrix derivative on the healing of periodontal defects in diabetic rats. J Periodontol. 2012 Nov 3.

One-sentence summary: Diabetes mellitus may produce a significant detrimental effect on bone density.

Running Title: Enamel matrix derivative treatment and Diabetes Mellitus.

ABSTRACT

Background: Diabetes Mellitus (DM) involves metabolic changes that can negatively influence periodontal tissues resulting in impaired periodontal repair. There is a lack of information about the outcomes of regenerative approaches under the influence of DM. Enamel matrix derivative (EMD) have been used in periodontal regenerative procedures resulting in improvement of clinical parameters. Thus, the aim of this histomorphometric study is to evaluate the healing of periodontal defects after treatment with EMD under the influence of DM.

Material and Methods: Twenty Wistar rats were randomly assigned to two groups: G1=DM was induced with a single intraperitoneal injection of streptozotocin (STZ) (n=10); G2= non-exposed to DM (n=10). Seven days after DM induction, bilateral fenestration defects were created at the buccal aspect of the first mandibular molar. After the surgeries the defects of each

animal were randomly assigned to two subgroups: non-treated control and treated with EMD. The animals were euthanized 21 days later and the percentage of defect fill, density of newly formed bone, and new cementum formation were histometrically assessed. The number of osteoclasts was determined by tartrate-resistant acid phosphatase. Weight and glucose were also analyzed. Mann Whitney test was used for comparison between groups and Wilcoxon for comparison between the start and end times (weigh and glucose) and between treatments (cementum formation and number of osteoclasts). One-way analysis of variance was used to the defect fill and bone density. Tukey test was used when the analysis of variance test detected significant differences. ($\alpha = 5\%$).

Results: G1 (DM) showed less defect fill and bone density (BD) compared to G2. EMD provided an increased defect fill in both groups and enhanced BD and new cementum formation only in G2. The number of tartrate-resistant acid phosphatase–positive osteoclasts was significantly higher in EMD-treated sites of G1.

Conclusion: DM may produce a significant detrimental effect on bone density. EMD may provide greater defect fill under diabetic or normal conditions; however, it may not significantly increase new cementum formation in diabetic animals.

KEY WORDS: Diabetes Mellitus; Enamel matrix proteins; Streptozocin; Rats; Regeneration.

INTRODUCTION

Diabetes Mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia due to absence of insulin or due to insulin inability to promote its effects. DM includes a clinically and genetically heterogeneous group of disorders that affect the metabolism of carbohydrates, lipids and proteins. [1, 2] It represents a public health problem due to its high prevalence, severe morbidity and mortality. According to the American Diabetes Association, [3] the current classification of DM is based on the pathophysiology of each form of the disease. The most common forms of DM include type 1 (results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas) and type 2 (includes individuals who have insulin resistance and usually have relative insulin deficiency). [3]

Metabolic changes occurring in DM and related pathological status like metabolic syndrome may influence the reparative capacity of periodontal tissues. *In vivo* studies have shown that DM can affect bone formation and remodeling and wound healing in rats. It was observed that the formation and deposition of bone matrix were diminished in diabetic rats. In addition, it was detected a reduced bone turnover by decreasing the production of osteoblasts, osteoclasts, osteoid and osteocalcin. [4, 5] Animal studies have demonstrated the negative impact of DM on the mechanical retention and osseointegration of implants. [5-11] The biological mechanisms that have been studied to validate and explain the relationship between DM and periodontal tissue alterations are: gingival microangiopathy, microbial changes, changes in host immune response, reduced production of collagen and increased collagenase, defective chemotaxis of polymorphonuclear leukocytes (PMN) [12 - 14] and production of advanced glycosylation end products (AGEs). [1, 15] According to Retzepi and Donus[16], suppressed differentiation, proliferation and/or bone forming capacity of osteoblastic cells during the

critical early healing period have been implicated as plausible pathogenetic mechanisms underlying poor bone formation in uncontrolled diabetes. These changes may slow healing ability and exacerbate the inflammatory response, which leads to reduced repair capacity.

Enamel matrix derivative protein (EMD) has been used in periodontal regenerative procedures due to its fundamental role in cementum development.[17] *In vitro* studies have demonstrated that EMD regulates bone formation through osteoblast and osteoclast cell proliferation.[18, 19] EMD also promotes periodontal ligament cell proliferation and increases the protein synthesis and mineral nodule formation by these cells.[20] The use of EMD in furcations and infra-osseous defects was evalutated in animal models, showing positive results.[21, 22] In addition, clinical trials showed significant improvement of clinical parameters in these defects with EMD application.[23, 24] A recent bayesian network meta-analysis comparing EMD, guided tissue regeneration and their combination therapies included 53 studies. The results showed that EMD, GTR and their combination therapies achieved a small additional treatment effect compared with flap operation, but the differences between combination and single therapies were generally small with wide intervals.[25]

It is recognized that a diabetic patient must require the control of DM before periodontal surgery. However, the optimal control of the condition may be challenging for some patients.[26] In addition, even with an approach to control glucose levels, it is possible that the induced cellular and tissue changes associated with DM may still be a factor to be considered. Several animal studies related the negative effect of DM on bone healing around titanium implants even with the insulin therapy. [27, 28 29] There is a paucity of information available from *in vivo* studies on whether DM would affect the healing capacity of periodontal tissues (periodontal ligament, bone, and cementum) after regenerative treatments. Therefore, the present study aims at

evaluating the possible influence of DM on the results of EMD treatment on a periodontal healing model in the absence of biofilm.

MATERIALS AND METHODS

ANIMALS

Twenty adult male Wistar rats (300–400 g) were included in the study. The animals were kept in plastic cages with access to food and water ad libitum. Prior to the surgical procedures all animals were allowed to acclimatize to the laboratory environment for a period of 5 days. The study protocol has been approved by the University of Campinas Institutional Animal Care and Use Committee.

EXPERIMENTAL DESIGN

The experimental design can be observed in Figure 1. The animals were randomly assigned (sealed, numbered and opaque envelopes) to one of the following treatment groups: G1 – diabetes mellitus (n=10) (DM) and G2 – non-exposed to DM (n=10). The animals of G1 were induced to DM on the first day of the experiment by an intraperitoneal injection of streptozotocin (STZ)[*]. An irreversible DM induction method was utilized as reported in other studies. [30, 31] The rats received a single intraperitoneal injection of 60 mg/kg STZ in 0.2 ml citrate buffer solution (0.01 M, pH 4.5). Whole blood glucose measurements were performed using puncture of the tail vein and measurement with a blood glucometer[†]. DM induction was performed following overnight fasting of 12 hours. The animals of G2 received a placebo

[*] Streptozotocin, sigma-Aldrich Chemie., St. Louis, MO, USA
[†] Accu-Chek Active, Roche, Jacarepaguá, RJ, Brazil

injection of citrate buffer solution. The changes in the blood glucose levels were observed 72 hours after the injections. DM induction was confirmed by the increase in the blood glucose level (≥ 250 mg/dL) measured during the period of the study (3 and 27 days) and the low body weight as described in the referred studies. [30, 31] Seven days after DM induction, general anesthesia was given by intramuscular administration of ketamine[‡] (0.5 ml/kg), an incision was made and a flap was raised at the base of the mandible. Periodontal fenestration defects (standardized with 4 mm in width, 3 mm in length and ≈ 1 mm deep) were created, bilaterally, by removing the superficial bone using a round dental bur (diameter 2 mm) at slow speed under saline irrigation. With a chisel, the distal root of the first mandibular molar was carefully denuded of its periodontal ligament, cementum and superficial dentin, avoiding excessive damage to the root that could compromise the pulp tissues and healing response. After the creation of the defects, they were randomly assigned (sealed, numbered and opaque envelopes) to two subgroups: non-treated control and treatment with EMD[§], following a split mouth design. EMD was directly applied into the defects with a syringe, without root surface conditioner. The soft-tissue defect was closed with separate sutures for the muscle and superficial skin. Neither antibiotics nor anti-inflammatory drugs were administered after the surgery. Acetaminophen[**] was given for pain control.

HISTOMETRIC PROCEDURE

Twenty-one days after defect creation, the animals were euthanized by perfusion under anesthesia. Subsequently, the jaws were removed and fixed in 4% buffered formalin for 24

[‡] Francotar, Virbac Laboratories, Roseira, SP, Brazil
[§] Emdogain, Straumann, Basel, Switzerland.
[**] Paracetamol, Abbott Laboratories, São Paulo, Brazil.

hours. The specimens were decalcified in a solution containing 17% EDTA, dehydrated in an ascending series of ethanol solution, embedded in paraffin and sectioned perpendicular to the long axis of the molar roots. Serial sections (6 μm) were transversally obtained in an apico-coronal direction and stained with hematoxylin and eosin. The coronal and apical margins of the defect were identified by the presence of a denuded first molar distal root. Ten sections per specimen representing the middle portion of the defect (24 μm apart) were selected, and the percentage of defect fill (DF), the density of newly formed bone (BD) and new cementum formation (NCF) were histomorphometrically assessed by a single blinded and calibrated examiner (MGC) with the aid of an image analysis system[††]. Both the percentage of bone density and defect fill were obtained by the point-counting technique.[32] Therefore, a square grid was overlaid on the defect area, constituting a drawing with intersections. The number of intersections under the presence of bone tissue (DF) or mineralized tissue (BD) was observed and counted. The extension of new cementum was obtained by linear measurement. The values were averaged and a mean value (in percentage) was obtained for each animal for statistical analysis. The percentage of new cementum (NCF) was obtained by the ratio of the extent of new cementum extension and the total extent of the instrumented root, multiplied by one hundred. [33]

STAINING FOR TRAP (ENZYMOHISTOCHEMISTRY)

Deparaffinized apico-coronal 6 μm-thick sections were incubated at 37° C for 15 min in a solution prepared by dissolving 4 mg of naphthol AS-BI[‡‡] and 24 mg of red violet salt[§§] in 30

[††] Image-Pro, Media Cybernetics, Silver Spring, MD.
[‡‡] Sigma Chemical Co., St Louis, MO, USA

mL of acetate buffer (pH 5.2) containing 0.3 mmol/L of tartrate[***] (pH 5.0;. Subsequently, sections were washed in distilled water and counterstained with hematoxylin. As a negative control for the TRAP activity, consecutive sections were incubated in substrate-free medium.[34] Two sections per specimen representing the middle portion of the defect were selected and quantitative analysis of the number of TRAP-positive cells was performed in the 1000 μm zone of the fenestration defect. The number of TRAP-positive cells was counted using a light microscope[†††] at a 40x magnification. The results are described as the percentage of TRAP-positive cells/mm^2.

STATISTICAL ANALYSIS

The variables weight and glucose did not meet the assumptions of parametric analysis by a statistical program[‡‡‡], neither with data transformation. Thus, it was used the nonparametric Mann Whitney test for comparison between groups and Wilcoxon for comparison between the start and end times. Regarding the defect fill and bone density, it was found that the data met the assumptions of a parametric analysis by the statistical program. Therefore, an One-way analysis of variance was used ($\alpha = 5\%$). Pair-wise multiple comparisons were carried out by the Tukey test when the one-way analysis of variance test detected significant differences. Regarding the extension of new cementum parameters and number of TRAP positive osteoclasts, it was found that the data did not meet the assumptions of a parametric analysis by the. Thus, they were

[§§] Sigma Chemical Co., St Louis, MO, USA
[***] Sigma Chemical Co., St Louis, MO, USA
[†††] Axioskop 2 plus®, Zeiss, Jena, Germany
[‡‡‡] PROC LAB SAS - Statistical Analysis System Software - version 8.2

analyzed by Mann Whitney test (α = 5%) for comparison between groups and Wilcoxon test (α = 5%) for comparison between treatments.

RESULTS

CLINICAL AND HISTOLOGICAL ANALYSIS

G1 animals (DM) had increased serum glucose levels (≥ 250 mg/dL) 72 hours after STZ injection and remained with high glucose levels throughout the experimental period. Statistical analysis revealed a significant difference between baseline and final glucose levels in G1 ($p \leq 0.05$) (Table 1). The animals in G1 (DM) showed lower weight than the animals in G2 animals (initial and final) (Table 1). G2 (non-exposed to DM) showed no alterations in general health, with a weight gain in the average levels expected for healthy rats and stable blood glucose levels, also within the normal range. Clinically, the wounds healed with no signs of infection and the animals continued to feed normally and gained weight. Primary wound closure was observed in all animals, and no ingrowth of oral or skin epithelium into the defect or surrounding tissue occurred (Figure 2). Histological analysis indicated newly formed bone and fibrous tissue scattered within the defect area. New cementum, when observed, occurred adjacent to existing cementum in a form of spicules or projections, being predominantly of cellular type (Figure 3). Ankylosis with obliteration of periodontal ligament space was not observed.

HISTOMETRIC ANALYSIS

The intra-group statistical analysis revealed a superior DF for the EMD-treated defects when compared to the non-treated controls for G1 (P=0.001) and G2 (P=0.002). The inter-group

analysis revealed a statistically significant difference between G1 and G2 for DF, with G1 showing less DF than G2, for the EMD-treated defects (P=0.0006) and non-treated controls (P=0.0002) (Table 2).

Regarding BD, the intra-group analysis showed that the difference between the EMD-treated sites and non-treated controls was not statistically significant for G1 (P=0.1458). In G2, the EMD-treated sites showed a superior BD when compared to non-treated controls (P=0.0001). The inter-group analysis revealed a statistically significant difference in BD between G1 and G2, in the EMD-treated sites (P<0.0001) and the non-treated controls (P<0.0001) (Table 3).

Considering NCF, the intra-group analysis showed a superior new cementum formation for the EMD-treated sites, only in G2 (P=0.0003). In G1, no statistically significant difference in NCF could be observed between EMD-treated sites and non-treated controls P=(0.1516). The inter-group analysis showed no statistically significant difference between G1 and G2, for EMD-treated sites (P=0.1184) and non-treated control (P=0.9839) (Table 4).

STAINING FOR TRAP

The intra-group analysis showed a significant difference between EMD-treated sites and non-treated control in the number of TRAP-positive cells in G1 (P=0.0051), with a greater value for the sites treated with EMD. In G2, no statistically significant difference was observed between EMD-treated sites and non-treated controls in the number of TRAP-positive cells (P=0.059). The intergroup analysis showed no statistically significant differences between G1 and G2, for EMD-treated sites (P=0.793) and non-treated control (P=0.3447) (Table 5) (Figure 5).

DISCUSSION

Metabolic changes occurring in DM and related pathological status like metabolic syndrome may influence the reparative capacity of periodontal tissues. [4 - 15] It is recognized that a diabetic patient must require the control of DM before periodontal surgery. However, the optimal control of the condition may be challenging for some patients.[26] In addition, it has been shown that even with an approach to control glucose levels, the alterations associated with DM may still be a factor to take into consideration [27-29] There is a paucity of information available from *in vivo* studies on whether DM would affect the healing capacity of periodontal tissues (periodontal ligament, bone, and cementum) after regenerative treatments.

Enamel matrix derivative protein is a topical agent that has been suggested as an alternative to enhance periodontal regeneration.[23] Therefore, the present study was designed to evaluate the possible influence of DM on the outcomes of enamel matrix derivative protein treatment using a periodontal healing model in the absence of biofilm.

The diabetogenic agent used was streptozotocin (STZ). STZ causes selective destruction of pancreatic β cells leading to a decrease in insulin production. It was observed a significant increase in plasma glucose levels (≥ 250 mg / dl) of animals treated with STZ, what is in accordance with other studies reported in the literature. [30, 35 – 38] The glucose level alteration was observed in the first 72 hours after induction and remained throughout the entire period of the experiment, showing the effectiveness of the induction model.

The rat model, as used by King's[39] group and modified by others,[40] seems to be a valuable model in studying periodontal healing without exposing the root surface to a dental biofilm. This acute model can be easily standardized and is relatively reproducible with respect to wound size, configuration and stability. It seems to be a predictable and reliable model to

study periodontal wound healing or regeneration for the specified period (21 days). Despite the several methodological advantages, there are important differences when compared to the human condition, like the absence of bacterial biofilm/epithelium and different healing potential between species. Therefore, caution should be taken when trying to extrapolate results from animal studies to a clinical setting.

The possible effect of DM on the results of EMD treatment has not been fully explored. It was observed that defect fill and bone density were significantly lower in G1 (DM), with or without EMD. It is recognized that bone metabolism is impaired in DM. Studies observed that osteocalcin is reduced in diabetic rats, indicating reduced number and function of osteoblasts. [7, 41] This model of DM induction (STZ), is characterized by low dynamic bone formation and osteoporosis. [5, 42] Shyng et al[43] showed that the extent of femoral cancellous bone surface covered by osteoid in the diabetic rats was reduced with low cancellous bone volume and trabecular thickness. In this study, the diabetic tibias had a significantly reduced length, dry weight, ash weight and calcium content compared with the control rats. Appositional bone formation was so low in the diabetic rat femurs that no calcein labelling in the femurs was observed. In the same way, animal studies have demonstrated the negative impact of DM on the mechanical retention and osseointegration of implants. [5 - 11] DM induces an impaired rate of bone formation around metal implants. [6] Histological studies suggest that diabetes may interfere with bone formation around implants. The newly formed bone around implants in diabetic rats were less organized, immature and of poorer quality than non-diabetic rats. [44] Thus, the reduced appositional bone formation rate would be a possible explanation for the decreased BD and DF. Besides, the data discussed above could help to explain the fact that EMD treatment was not able to enhance BD in G1 (DM).

The present study showed greater defect fill with EMD treatment in the presence or absence of DM. EMD plays an important role in cell proliferation and in growth factor production. [45 - 47] Amelogenin is the major component of EMD (> 90%). This protein is important in biomineralization and hard tissue formation.30 Data from Bosshardt's study [47] (where 103 papers were analyzed about EMD biological effect) showed that EMD treatment may have direct effects on cell attachment, spreading, and chemotaxis; cell proliferation and survival; expression of transcription factors; expression of growth factors, cytokines, extracellular matrix constituents, and other macromolecules; and expression of molecules involved in the regulation of bone remodeling. Additionally, EMD may have osteopromotive effects. [48]

It was observed an enhanced NCF with EMD treatment only in G2 (non-exposed to DM). These findings are in agreement with other studies using the same animal model that showed NCF after regenerative procedures in non-diabetic animals. [39, 40, 49, 50] Enhanced NCF promoted by EMD treatment suggests an effect of these proteins in cementoblast precursor cells or in cementoblast cells itself. Tokiyasu et al.[51] observed that EMD has the ability to promote some activities in mesenchymal derived cells. EMD promoted proliferation of 3 cell lines (one cementoblast and two osteoblast cell lines) and altered gene expression of all cell types. Besides, Lyngstadaas et al.[45] suggested that amelogenin is crucial in enamel formation and biomineralization. These results may help to explain the NCF in the present study. In this study, as well as in a previous study of our group[33], new cementum was confined to the margins of the exposed root surface and formed spicules arching toward the wound area, remaining contiguous with the preexisting cementum surface. This finding is in agreement with other studies[39, 50, 52] that showed a limited NCF in this specific model, with or without regenerative treatment. It can

be recognized that the cementum formation is limited, even in the absence of previous exposure to a plaque biofilm or a systemic factor like DM. EMD treatment was not able to enhance NCF in G1 (DM). Gokhan et al.[53] reported on the average thickness of cementum layer on decalcified teeth extracted from non-diabetic patients compared to the average thickness of cementum layer from type 2 diabetic patients. A significant difference was observed between diabetic and non-diabetic patients with regard to the thickness of the cementum layer. Diabetic patients presented thinner cementum layer at the central part of the root and at the midpoint of the coronal half. A study about the effect of STZ on the secretory ameloblasts of rat incisors suggested that there was a pronounced inhibition in the movement of secretion granules into Tomes' process, as well as an ectopic secretion of the enamel matrix protein.[54] It can be hypothesized that DM may interfere in cell response (proliferation and gene expression) and consequently with EMD effect. There is a need for more studies designed to explain the mechanism by which DM would affect cementoblasts and their response to regenerative approaches.

In the present study, the defects treated with EMD showed an increased number of TRAP-positive cells when compared to non-treated defects in G1 (DM). Otsuka et al.[48] and Itoh et al.[55] observed osteoclast differentiation through RANKL production by osteoblasts. The authors concluded that EMD is able to stimulate osteoclasts as well as osteoblasts, suggesting that it provides a local environment favorable to bone remodeling activities. In addition, Reseland et al.[56] observed that EMD stimulates osteoblast differentiation and the expression of several mRNAs and proteins suggesting a secondary osteoclast-stimulating effect. Thus, these results could help to explain the observation of higher number of TRAP-positive cells coupled with a greater defect fill after EMD treatment. The number of TRAP-positive cells was not statistically different when G1(DM) was compared to G2. This result is in accordance with

Shyng' s study. [43] They did not observe any histological evidence of an increased osteoclastic resorption rate when compared diabetics rats to non-diabetics and insulin-treated rats.

Within the limits of the present study, the following conclusions may be drawn:

1- DM may negatively interfere with the filling of periodontal fenestration defects in the rat model,
2- EMD may provide increased defect filling under diabetic or normal conditions,
3- DM negatively interferes with the proportion of mineralized tissue (BD) after healing of periodontal fenestration defects in the rat model,
4- EMD may not increase BD in the presence of DM,
5- EMD may not significantly increase new cementum formation in periodontal fenestration defects of diabetic animals.
6- More studies are necessary to elucidate the influence of DM on the outcomes of different regenerative aproches.

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TABLES

Table 1 – Mean ± SD of weight (g) and blood glucose levels (mg/dl) of non-diabetics and diabetics animals in the different groups and experimental periods.

GROUPS	Initial weight (g)	Final weight (g)	Initial glucose (mg/dl)	Final glucose (mg/dl)
G1	266.74 ±26.17 Aa	282.12 ± 70.87 Ab	356.1±45.04 Aa	471±41.08 Ab
G2	355.66 ±31.28 Ba	448.20±39.88 Bb	128.9±11.41 Ba	132.7±10.95 Bb

Different capital letters in the column (between groups) and different non-capital letter in the line (between periods) represent statistically significant difference ($p \leq 0.05$).

Table 2 - Mean ± SD (%) of defect fill 21 days after the creation of the defects for all the Experimental groups

GROUPS	TREATED WITH EMD	NON-TREATED CONTROL	P-VALUE
G1	69.14 ± 12.76 Ba	64.82 ± 13.18 Bb	0.0010
G2	97.54 ± 0.86 Aa	96.49 ± 1.18 Ab	0.0020
P-VALUE	0.0006	0.0002	

Different capital letters in the column and different non-capital letter in the line represent statistically significant difference by ANOVA / Tukey test ($p < 0.05$).

Table 3 - Mean ± SD (%) of bone density 21 days after the creation of the defects for all the experimental groups

GROUPS	TREATED WITH EMD	NON-TREATED CONTROL	P-VALUE
G1	10.96 ± 4.12 Ba	9.55 ± 2.56 Ba	0.1458
G2	69.23 ± 4.82 Aa	55.78 ± 5.68 Ab	0.0001
P-VALUE	<0.0001	<0.0001	

Different capital letters in the column and different non-capital letter in the line represent statistically significant difference by ANOVA / Tukey test ($p < 0.05$).

Table 4 - Mean ± SD (%) of new cementum formation 21 days after the creation of the defects for all the experimental groups

GROUPS	TREATED WITH EMD	NON-TREATED CONTROL	P-VALUE
G1	9.62± 1.69 Aa	7.69± 2.41 Aa	0.1516
G2	15.06± 7.74 Aa	8.16± 4.62 Ab	0.0003
P-VALUE	0.1184	0.9839	

Different capital letters in the column and different non-capital letter in the line represent statistically significant difference by ANOVA / Tukey test ($p < 0.05$).

Table 5 - Mean ± SD of number of TRAP-positive cells/mm2 in a 1000 µm zone on fenestration defects

GROUPS	TREATED WITH EMD	NON-TREATED CONTROL	P-VALUE
G1	16.89± 5,21 Aa	12.11± 4.16 Ab	0.0051
G2	15.30± 6,10 Aa	10.20± 2.49 Aa	0.0593
P-VALUE	0.793	0.3447	

Different capital letters in the column mean statistically significant difference by Mann Whitney test ($\alpha = 0.05$). Different non-capital letter in the line represent statistically significant difference by wilkoxon test ($\alpha = 0.05$).

FIGURE LEGENDS

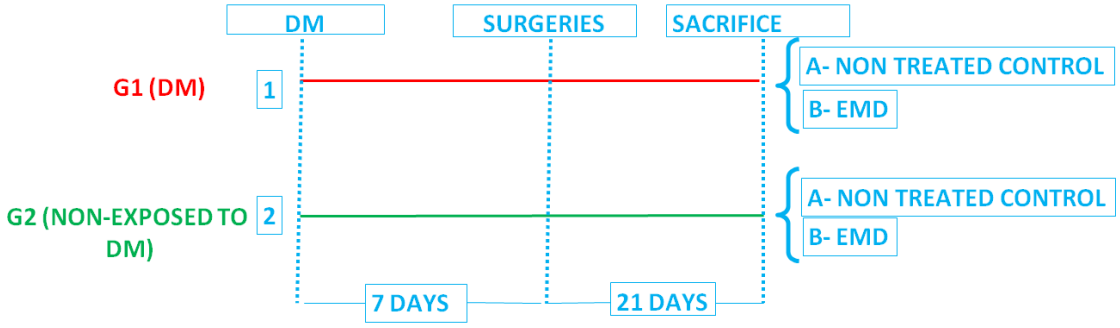

Figure 1: Schematic illustration of the experimental design.

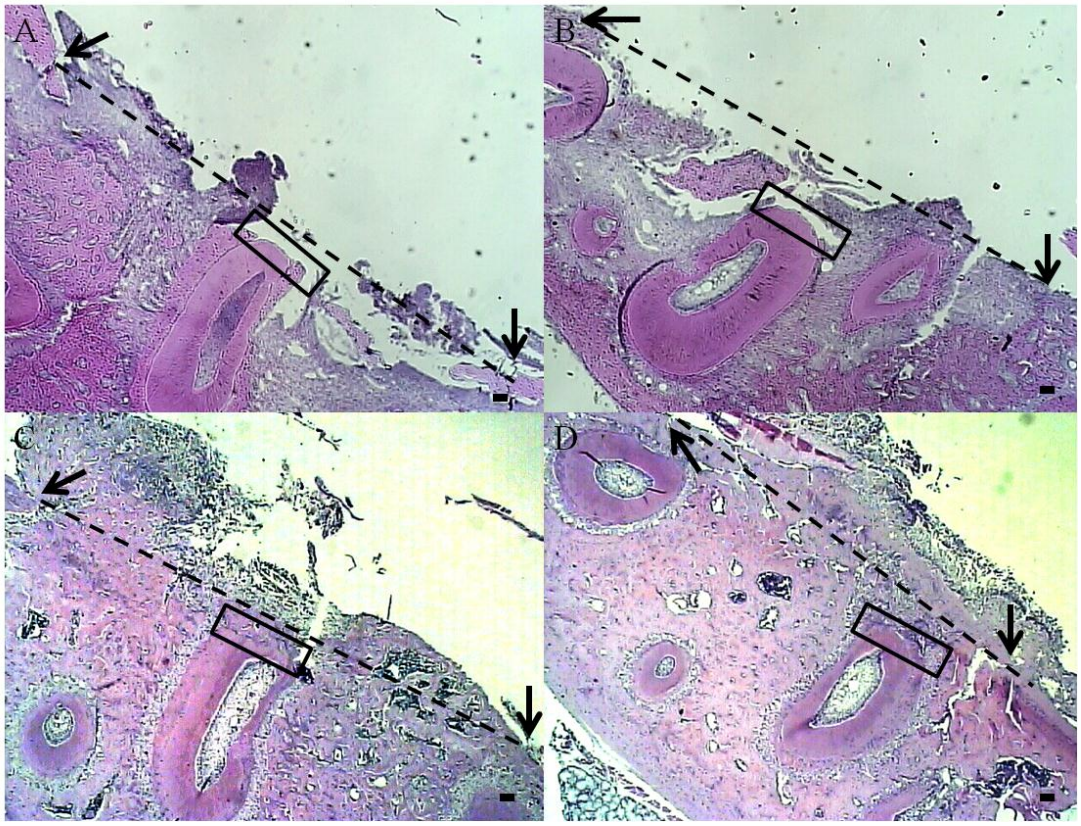

Figure 2: Histological illustration of periodontal healing 21 days after the surgical creation of the fenestration-type defects (H&E, original magnification x 5). A) G1 (DM), non-treated control. B) G1 (DM), treated with EMD. Bar = 1,000 µm. C) G2 (Non-exposed to DM), non-treated control. D) G2 (Non-exposed to DM), treated with EMD. Bar = 1,000 µm. Lines correspond to the original extension of the defects. These lines were drawn using the external edges (arrows indicate edges) of the defect as a reference. Rectangles indicate root instrumented areas.

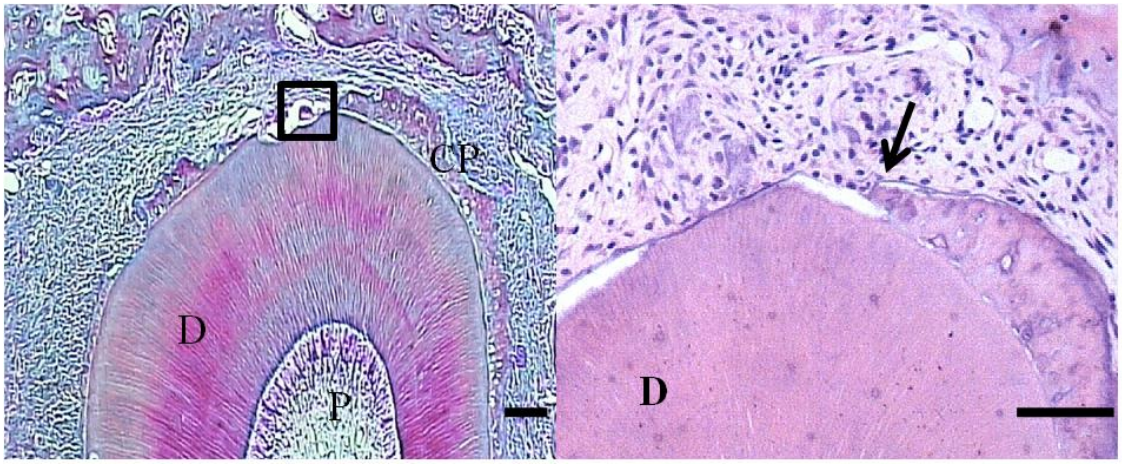

Figure 3: Histologic illustration of new cementum formation. A) Rectangle indicates new cementum formation (Mallory, original magnification x 20). Bar = 100 µm. B) Arrow indicates the new cellular cementum formation area (H&E, original magnification x 50). Bar = 20 µm. D = dentin; CP = preexisting cementum; P = pulp.

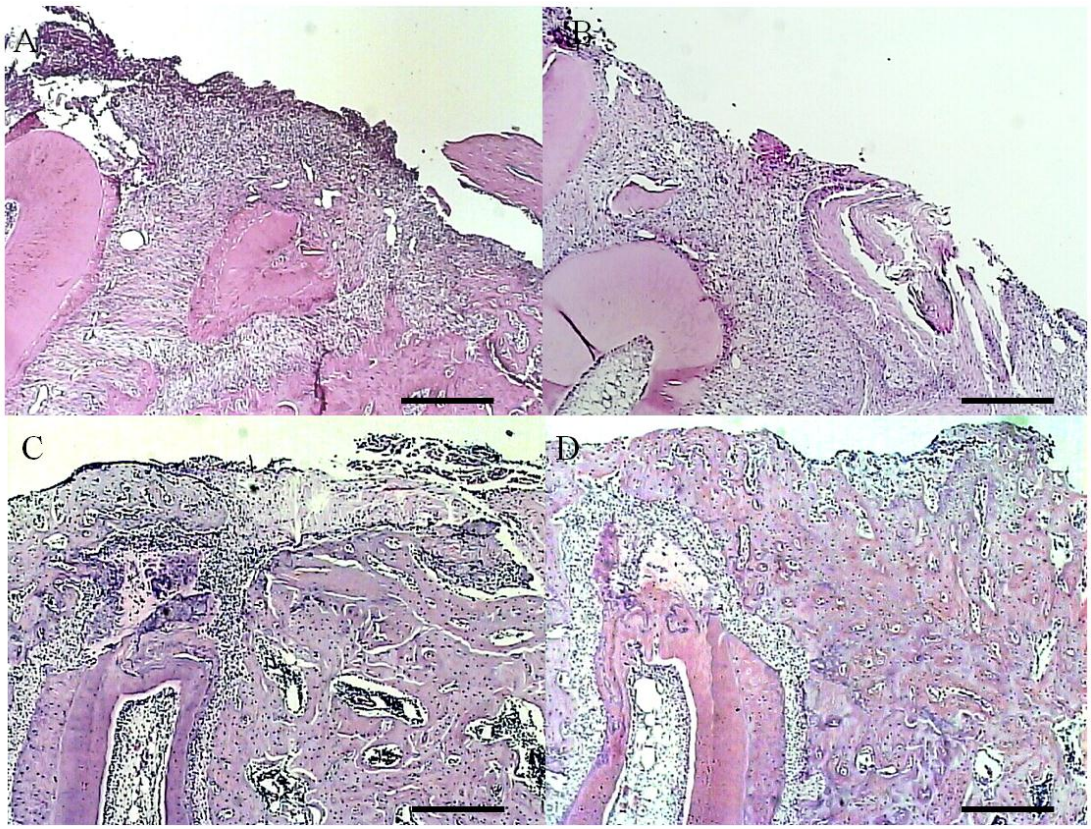

Figure 4: Histologic illustration of bone density. (H&E, original magnification x 10). Bar = 50 µm. A) G1 (DM), non-treated control. B) G1 (DM), treated with EMD. C) G2 (Non-exposed to DM), non-treated control. D) G2 (Non-exposed to DM), treated with EMD.

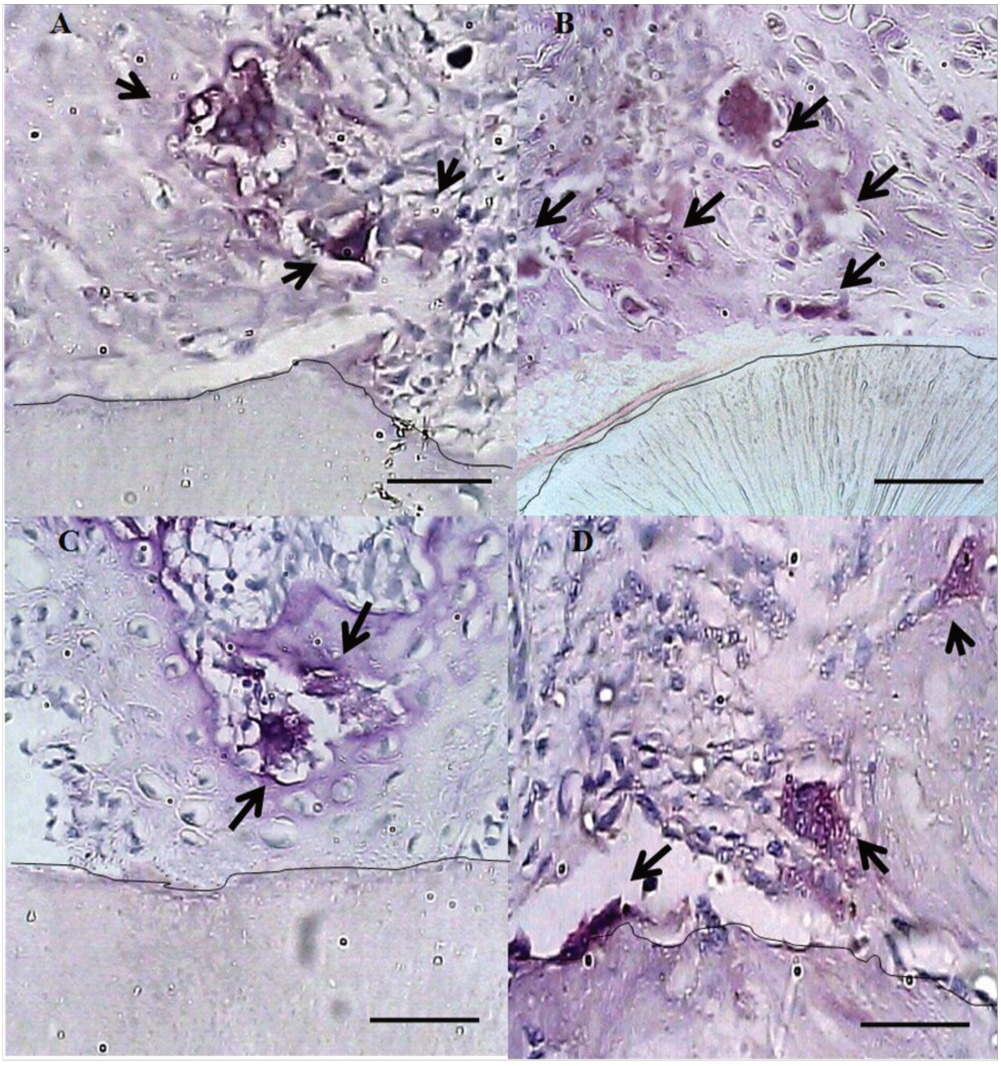

Figure 5: Histological illustration of the histological aspects of TRAP-positive cells (TRAP, original magnification x 20). A) G1 (DM), non-treated control. B) G1 (DM), treated with EMD Bar = 100µm. C) G2 (Non-exposed to DM), non-treated control. D) G2 (Non-exposed to DM), treated with EMD. Bar = 100 µm. Arrows indicate TRAPpositive cells. Lines represent root instrumented areas.

CAPÍTULO 2

Impact of chronic stress on the outcome of enamel matrix derivative treatment in rats: histometric analysis.

One-sentence summary: Chronic stress may produce a significant detrimental effect on bone density with or whitout enamel matrix treatment

Running Title: Chronic stress influence on enamel matrix derivative treatment.

ABSTRACT

Background: Psychological stress and clinical hypercortisolism have been related to direct effects on bone metabolism. However, there is a lack of information regarding the outcomes of regenerative approaches under the influence of chronic stress (CS). Enamel matrix derivative (EMD) have been used in periodontal regenerative procedures resulting in improvement of clinical parameters. Thus, the aim of this histomorphometric study is to evaluate the healing of periodontal defects after treatment with EMD under the influence of CS in the rat model.

Material and Methods: Twenty Wistar rats were randomly assigned to two groups: G1= CS – chronic stress (restraint stress for 12 hours/day) (n=10), G2= non-exposed to CS (n=10). Fifteen days after initiation of CS, fenestration defects were created at the buccal aspect of the first mandibular molar of all animals from both groups. After the surgeries the

defects of each animal were randomly assigned to two subgroups: non-treated control and treated with EMD. The animals were euthanized 21 days later.

Results: G1 showed less bone density (BD) compared to G2. EMD provided an increased defect fill (DF) in G1 and higher BD and new cementum formation (NCF) in both groups. The number of tartrate-resistant acid phosphatase–positive osteoclasts was significantly higher in G1 when compared to G2 and in EMD-treated sites of both groups.

Conclusion: Chronic stress may produce a significant detrimental effect on bone density. EMD may provide greater defect fill in the presence of CS and increased bone density and cementum formation in the presence or absence of CS.

KEY WORDS: Animal studies; chronic stress; Enamel matrix proteins; Rats; Regeneration.

INTRODUCTION

Stress was defined as a state of response of the body to forces acting simultaneously in the body and, if excessive (when surpasses adaptive capacity) leads to disease and possibly disease exhaustion and death. [1] Periodontitis has been related to stress and psychological factors. A systematic review evaluated the available scientific evidence regarding the relationship between periodontitis and stress. Fourteen articles (seven case-control studies, six cross-sectional studies, and one prospective clinical trial) were included in the analysis. Among the selected articles, 57.1% found a positive outcome between

psychosocial factors/stress and periodontal disease, 28.5% observed a positive outcome for some characteristics and a negative outcome for others, and 14.2% found a negative outcome. Thus, stress, distress, and inadequate coping behaviors are important risk indicators for periodontal disease. [2]

Some animal studies demonstrated less attachment and more alveolar bone loss after exposing the experimental animals to restraint stress [3 -7 7] Breivik et al. [8] and Semenoff Segundo et al.,[6] demonstrated that periodontal disease susceptibility and progression could be explained in part by brain-neuroendocrine-immune regulatory mechanisms. Hypothalamus-pituitaryadrenal (HPA) axis hyper-activation seems to play an important role in the increased periodontal disease susceptibility. [6, 8] Clinical studies [9 - 12] established that HPA axis hyperactivation may increase the odds ratio for periodontal disease. Gameiro et al. [13] investigated the effect of systemic stress on the biological reactions occurring during orthodontic tooth movement. They found that persistent systemic stress during long time increased bone resorption during orthodontic tooth movement as well as the number of osteoclasts. Likewise, Vandevska-Radunovic & Murison [14] observed that rats under emotional stress had the smallest amount of cellular cementum.

Enamel matrix derivative protein (EMD) has been used in regenerative periodontal procedures due to its key role in cementum development. [15] *In vitro* studies have demonstrated that EMD regulates bone formation by inducing proliferation of osteoblasts and osteoclasts. [16, 17] EMD also promotes proliferation of periodontal ligament and increases protein synthesis and mineral nodule formation by these cells. The use of EMD in furcations and infra-bony defects was evaluated in animal models, showing positive results.

[19, 20] Furthermore, a recent systematic review supports the positive effect of the use of EMD in furcations and infra-bony defects. [21] The use of EMD under behavioral and systemic factors has been suggested as an alternative to improve the repair of periodontal tissues. [22, 23]

There is a lack of information from in vivo studies on the possible effect of chronic stress on the healing capacity of periodontal tissues after regenerative treatments. Therefore, the present study aims at evaluating the possible influence of chronic stress on the results of EMD treatment on a periodontal healing model in the absence of biofilm.

MATERIALS AND METHODS

ANIMALS

Twenty adult male Wistar rats (300–400 g) were used. The animals were kept in plastic cages with food and water *ad libitum*. The study protocol has been approved by the University of Campinas Institutional Animal Care and Use Committee.

EXPERIMENTAL DESIGN

The experimental design can be observed in Figure 1. The animals were randomly assigned to one of the following treatment groups: G1= CS – chronic stress (restraint stress for 12 hours/day) (n=10), G2= non-exposed to CS (n=10). G1 was subjected to restraint stress as previously described.[24] Briefly, restraint stress was applied daily from 8:00 pm to 8:00 am (12 hours) for fifteen days preoperatively and 21 days postoperatively with the use of a flexible plastic mesh (30 · 30 cm) and the animals housed

in plastic pipes (10 · 30 cm) full of holes for restraint and isolation purposes. G1 animals were incapable to move and had no access to food or water during the period of restraint. G2 animals were pair feeding (limited access to food and water) but without restraint. At the end of the daily 12-hour cycle, all animals were returned to their cages. Fifteen days after the beginning of CS, G1 and G2 animal were subjected to the creation of the fenestration defects as previously described. [22, 23] Periodontal fenestration defects (4 mm in width, 3 mm in length and ≈ 1 mm deep) were created, bilaterally, by removing the superficial bone with a round dental bur (diameter 2 mm) at slow speed under saline irrigation. With a chisel, the distal root of the first mandibular molar was carefully denuded of its periodontal ligament, cementum and superficial dentin. [22, 23] After defects creation, they were randomly assigned to two subgroups: non-treated control and treatment with EMD[*], following a split mouth design. EMD was directly applied into the defects with a syringe, without root surface conditioner. The soft-tissue defect was closed with separate sutures for the muscle and superficial skin. Antibiotics and anti-inflammatory drugs were not administered after surgery. Acetaminophen[†] was given for pain control.

HISTOMETRIC PROCEDURE

The euthanasia was performed twenty-one days after defect creation by perfusion under anesthesia. The jaws were removed and fixed in 4% buffered formalin for 24 hours and subsequently decalcified in a solution containing 17% EDTA. After demineralization, the specimens were dehydrated in an ascending series of ethanol solution,

[*] Emdogain, Straumann, Basel, Switzerland.
[†] Paracetamol, Abbott Laboratories, São Paulo, Brazil.

embedded in paraffin and sectioned perpendicular to the long axis of the molar roots. Serial sections (6 μm) were transversally obtained in an apico-coronal direction and stained with hematoxylin and eosin. The coronal and apical margins of the defect were identified by the presence of a denuded first molar distal root. Ten sections per specimen representing the middle portion of the defect (24 μm apart) were selected, and the percentage of defect fill (DF), the density of newly formed bone (BD) and new cementum formation (NCF) were histomorphometrically assessed by a single blinded and calibrated examiner (MGC) with the aid of an image analysis system[‡]. Both the percentage of bone density and defect fill were obtained by the point-counting technique.[22, 23] Thus, a square grid was overlaid on the defect area, constituting a drawing with intersections. The number of intersections under the presence of bone tissue (DF) or mineralized tissue (BD) was observed and counted. The extension of new cementum was obtained by linear measurement. The values were averaged and a mean value (in percentage) was obtained for each animal for statistical analysis. The percentage of new cementum (NCF) was obtained by the ratio of the extent of new cementum extension and the total extent of the instrumented root, multiplied by one hundred. [22, 23]

STAINING FOR TRAP (ENZYMOHISTOCHEMISTRY)

As previously described, [22, 23] staining for TRAP were processed using deparaffinized apico-coronal 6 μm-thick sections incubated at 37°C for 15 min in a solution prepared by dissolving 4 mg of naphthol AS-BI[§] and 24 mg of red violet salt[**] in 30 mL of

[‡] Image-Pro, Media Cybernetics, Silver Spring, MD.
[§] Sigma Chemical Co., St Louis, MO, USA

acetate buffer (pH 5.2) containing 0.3 mmol/L of tartrate[††] (pH 5.0). Subsequently, sections were washed in distilled water and counterstained with hematoxylin. As a negative control for the TRAP activity, consecutive sections were incubated in substrate-free medium.[25] Two sections per animal, representing the middle portion of the defect, were selected and quantitative analysis of the number of TRAP-positive cells was performed in the 1000 μm zone of the fenestration defect. The number of TRAP-positive cells was counted using a light microscope[‡‡] at a 40x magnification. The results are described as the percentage of TRAP-positive cells/mm^2.

STATISTICAL ANALYSIS

After exploratory data analysis, the analysis of variance test was applied in a split plot design. Pair-wise multiple comparisons were carried out by the Tukey test when the ANOVA test detected significant differences. A significance level of 0.05 was set for all statistical comparisons.

RESULTS

CLINICAL AND HISTOLOGICAL ANALYSIS

Animals had the defects healed with no signs of infection and continued to feed normally. The wounds of all animals had primary wound closure and no ingrowth of oral or skin epithelium into the defect or surrounding tissue occurred (Figure 2). Histological analysis indicated newly formed bone and fibrous tissue scattered within the defect area.

[**] Sigma Chemical Co., St Louis, MO, USA
[††] Sigma Chemical Co., St Louis, MO, USA
[‡‡] Axioskop 2 plus®, Zeiss, Jena, Germany

New cementum, was limited and when observed occurred adjacent to existing cementum in a form of spicules or projections, being predominantly of cellular type.

HISTOMETRIC ANALYSIS

The intra-group statistical analysis revealed a superior DF for the EMD-treated defects when compared to the non-treated controls only for G1. The inter-group analysis revealed a statistically significant difference between G1 and G2 for DF, with G1 showing less DF than G2, only for the non-treated control (Table 1).

The EMD-treated sites showed a superior BD when compared to non-treated controls for G1 and G2, after the intra-group analysis. The inter-group analysis revealed a statistically significant difference in BD between G1 and G2, in the EMD-treated sites and the non-treated controls (Table 2), with G1 showing inferior values.

Considering NCF, the intra-group analysis showed a superior new cementum formation for the EMD-treated sites in G1 and in G2. The inter-group analysis showed statistically significant difference between G1 and G2, for EMD-treated sites only. In non-treated control, no statistically significant difference between G1 and G2 was observed (Table 3).

TRAP STAINING

The intra-group analysis showed a significant difference between EMD-treated sites and non-treated control in the number of TRAP-positive cells in G1 and in G2, with a greater value for the sites treated with EMD. The intergroup analysis showed statistically

significant differences between G1 and G2, for EMD-treated sites and non-treated control (Table 4).

DISCUSSION

Psychological stress and clinical hypercortisolism have been related to direct effects on bone metabolism, such as reduced bone mineral density [26] and inhibited osteoblast ativity. [27, 28] Physiological stimulation of the sympathetic nervous system modulates bone metabolic activity - in vivo - as evaluated by expression of IL-6 mRNA in calvaria. [29] Enamel matrix derivative protein is a topical agent that has been suggested as an alternative to enhance periodontal regeneration.[15] There is a lack of information from in vivo studies on the possible effect of chronic stress on the healing capacity of periodontal tissues (periodontal ligament, bone, and cementum) after regenerative treatments. Therefore, the present study aims at evaluating the possible influence of chronic stress on the results of EMD treatment on a periodontal healing model in the absence of biofilm.

The experimental model of chronic stress was based in a previous study[24] and in studies that showed the negative effect of restraint stress on the response of induced periodontitis in rats [5, 6, 30] As nocturnal animals, it was shown that the effect of the stressor agent may be enhanced significantly if applied at this time. [31] In this study, the concentration values of systemic biomarkers of stress (corticosterone and catecholamine: adrenaline and noradrenaline) were not assessed. However, it has been previously shown that this chronic stress exposure regimen (restraint stress applied daily from 8:00 pm to

8:00 am - 12) may promote plasma levels of corticosterone and catecholamines significantly higher than the non-restraint groups. [24]

The fenestration model was initially used by King[32] and modified by other authors. [33] It seems to be a useful model to study periodontal healing without biofilm exposure. This acute model can be easily standardized and is relatively reproducible in relation to configuration, size and stability. It seems to be a predictable and reliable model to study periodontal wound healing or regeneration for the specified period (21 days). Despite the several methodological advantages, there are significative differences when compared to the human condition: absence of bacterial biofilm / epithelium and healing potential different between species. Consequently, caution should be taken when trying to extrapolate results from animal studies to a clinical reality.

This study showed greater defect fill with EMD treatment in the presence of CS. EMD stimulates the growth of multiple mesenchymal cell types including fibroblasts, cementoblasts, and osteoblasts. [15, 34-37] The major constituent of EMD is amelogenin. [34] Izumikawa et al.[38] demonstrated that amelogenin promoted the differentiation and mineralization of rat bone marrow stem cells. EMD promoted gene expression of various osteoblast differentiation markers including a number of collagen types and isoforms, SMAD intracellular proteins, osteopontin, cadherin, alkaline phosphatase, and bone sialoprotein. [39] EMD also upregulated a variety of growth factors including bone morphogenetic proteins, vascular endothelial growth factors, insulin-like growth factor, transforming growth factor, and their associated receptor proteins. Bosshardt[36] analyzed all available biological data (103 papers) about EMD effect at the cellular and molecular levels that are relevant in the context of periodontal wound healing

and tissue formation. The analysis showed that EMD treatment may have direct effects on cell attachment, spreading, and chemotaxis; cell proliferation and survival; expression of transcription factors; expression of growth factors, cytokines, extracellular matrix constituents, and other macromolecules; and expression of molecules involved in the regulation of bone remodeling. These data could help to explain the greater DF and BD achieved with EMD treatment.

In this study, EMD did not enhanced DF in G2. Although EMD treatment did not increase the DF in G2, it is important to mention that the mean values of DF are high in both subgroups of the CS and non-exposed group. This result differs from the findings of previous studies of our group, [22, 23] showing greater DF in non-exposed groups. It can be assumed that the biological healing response may be different among different groups of animals in spite of the fact that the mean values of DF are comparable to those of previous studies.

DF was significantly lower in G1, when compared to G2 in the non-treated control sites. Peruzzo et al [24] reported increased levels of IL- 1β, IL-6 and IFN-γ associated with stress increased bone loss resulting from ligature-induced periodontitis. In addition, reductions in IL-1 and -8 were found in the fluid from blister wounds in patients exhibiting higher stress scores compared to controls. [40] Moreover, studies in rodents demonstrated that restraint stress significantly decreased wound healing. [41, 42] Oxygen homeostasis is critical to all phases of wound healing and restraint stress can further promote wound hypoxia.[43] Restraint-stressed mice had higher levels of inducible nitric oxide syntheses levels, an indicator of wound hypoxia at the wound site, when compared with controls.[44] Besides, psychological stress may reduce cell infiltration at the wound site. Mice subjected to restraint stress had less leukocyte infiltration to the wound sites than control mice at 1 and 3 days after wounding.[44] Matrix metalloproteinase

(MMP) enzymes are important in repair process and stress can down regulate MMP production. In a human study using the blister wounds model, there was a negative correlation between plasma cortisol levels and MMP-2 protein levels at the wound site. [45] In addition, mice subjected to stress had fewer activated MMP-2 and MMP-9 after wounding, compared with control mice. [46]

Bone density was significantly lower in G1, with or without EMD. It has been shown that cortisol has direct effects on bone, and clinical hypercortisolism is consistently associated with reduced bone mineral density. [26] Cortisol reduces the utilization of amino acids for protein formation in muscle cells. Stress resulting in cortisol excess can lead to progressive protein loss, muscle weakness and atrophy, and bone mass loss through increased calcium excretion and less calcium absorption. [47] A human study evaluated the hypothesis that cortisol mediates the relationship between bone density and depression in postmenopausal women. There were significant inverse relationships between salivary cortisol values and bone density scores at every measured bone site. Mediation analyses suggest that 51 – 67% of the association between depression and bone density could be attributed to stress-induced changes in cortisol. [48] An animal study showed that different kinds of stress affect mandible bone mass of Wistar rats. [49] Stress-induced hypercortisolism and the excess of released glucocorticoids also directly affect bones, inhibiting osteoblastic activity and causing osteoporosis. [27, 28] The present findings regarding BD are in agreement with the findings of Patterson-Buckendahl et al. that observed decreased mandible BD after restraint stress. [50]

It was observed an enhanced NCF with EMD treatment in the groups of the present study, with or without exposure to CS. These findings corroborate the results of other studies using the same animal model that showed NCF after regenerative procedures. [32, 33, 51, 52] The

results regarding NCF (enhanced by EMD treatment) suggest an effect of EMD in cementoblast precursor cells or in cementoblast cells itself. Human PDL fibroblasts showed some morphologic changes that made them more similar to cementoblasts than to fibroblasts, suggesting a process of cellular differentiation under EMD action.[53] EMD has the ability to promote some activities in mesenchymal derived cells promoting proliferation of 3 cell lines (one cementoblast and two osteoblast cell lines) and altered gene expression of all cell types. [54] In addiction, it was suggested that amelogenin is crucial in enamel formation and biomineralization. [34] In this study, as well as in previous studies of our group, [22, 23] new cementum was confined to the margins of the exposed root surface and formed spicules arching toward the wound area, remaining contiguous with the preexisting cementum surface. This finding is in agreement with other studies [32, 51, 55] that showed a limited NCF in this specific model, with or without regenerative treatment. It can be accepted that the cementum formation is limited, even in the absence of previous exposure to a plaque biofilm or a modifier factor like CS. In this study, the subgroups non treated with EMD presented NCF. Accordingly, Huang et al. [52] demonstrated NFC without any regenerative treatment in this model. In the present study it was also observed that CS decreased NCF in G1, when compared to G2 in the non-treated control sites. Vandevska-Radunovic & Murison [14] investigate the effect of emotional stress on apical root resorption and tooth displacement during orthodontic tooth movement in rats. They found that rats under emotional stress had the smallest amount of cellular cementum and showed significant reduction in tooth displacement in the earlier periods. Longer periods of stress caused greater tooth movement when compared to control. Animal under stress also showed the greatest amount of root resorption. There is a need for more studies designed to

explain the mechanism by which CS would affect cementoblasts and their response to regenerative approaches.

In the present study, the defects treated with EMD showed an increased number of TRAP-positive cells when compared to non-treated defects in G1 and G2. EMD can induce osteoblasts cells to produce RANKL and stimulate osteoclast differentiation, what suggests that EMD is able to stimulate osteoclasts as well as osteoblasts, providing a local environment favorable to bone remodeling activities. [37, 56] Accordingly, EMD stimulates osteoblast differentiation and the expression of several mRNAs and proteins suggesting a secondary osteoclast-stimulating effect. [35] Bioactive factors released from EMD stimulate osteoblasts producing TGF-β1 and IL-6. TGF-β1 enhances osteoblasts proliferation and differentiation and inhibits osteoblasts apoptosis. IL-6 promotes osteoblasts function by activating proliferation and differentiation and on the other hand activates the transforming of osteoclast progenitors into osteoclasts to accelerate bone remodeling. [57] The number of TRAP-positive cells was significantly higher when G1 was compared to G2 in both subgroups (EMD treated sites and non-treated control sites). Gameiro et al.[13] investigated the effect of systemic stress on the biological reactions occurring during orthodontic tooth movement. They found that persistent systemic stress increased bone resorption during orthodontic tooth movement as well as the number of osteoclasts. Some evidences[58, 59] suggest that epinephrine can increase the expression of osteotrophic factors, such as interleukin (IL)-6, IL-11, PGE2 and RANKL; as well as the formation of osteoclast-like cells from mouse bone marrow cells by activating adrenoceptors. [59] Peruzzo et al. [24] verified increased levels of mRNA levels of proresorptive factor as IL-1β, IL-6 e RANKL during chronic stress. An *in vivo* study showed that activation of adrenoceptors on osteoblastic cells can stimulate bone resorption in intact mouse calvaria. [59, 60] Kondo & Togari

[61] demonstrated that restraint stress increased IL-6 mRNA expression via peripheral epinephrine from the adrenal glands, what suggests that the activity of sympathetic nerves has a significant effect on osteoclastogenesis by modulation of the expression of osteotrophic factors in osteoblastic cells. These results can provide a biologic basis for the higher number of TRAP-positive osteoclast cells in G1, compared to the non-exposed group and also the reduced BD in the same group.

Within the limits of the present study, the following conclusion may be drawn:

1- Chronic stress may produce a significant detrimental effect on bone density.

2- EDM may provide greater defect fill when compared to a non-treated control in the presence of chronic stress

3- EMD may increase bone density and new cementum formation in the presence or absence of chronic stress.

4- More studies are necessary to elucidate the influence of CS on the outcomes of different regenerative approaches and explain its mechanism.

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TABLES

Table 1 - Mean ± SD (%) of defect fill 21 days after the creation of the defects for all the Experimental groups

GROUPS	TREATED WITH EMD	NON-TREATED CONTROL
G1	**96.54± 1.38 Aa**	**93.36± 2.54 Bb**
G2	**97.07± 0.41 Aa**	**96.98± 1.12 Aa**

Means followed by different letters (capital letters horizontally and different non-capital letter vertically) represent statistically significant difference by ANOVA / Tukey test ($p < 0.05$).

Table 2 - Mean ± SD (%) of bone density 21 days after the creation of the defects for all the experimental groups

GROUPS	TREATED WITH EMD	NON-TREATED CONTROL
G1	**41,24± 2,92 Ab**	**32,36±4,77 Bb**
G2	**71,52± 4,86 Aa**	**67,06± 5,09 Ba**

Means followed by different letters (capital letters horizontally and different non-capital letter vertically) represent statistically significant difference by ANOVA / Tukey test ($p < 0.05$).

Table 3 - Mean ± SD (%) of new cementum formation 21 days after the creation of the defects for all the experimental groups

GROUPS	TREATED WITH EMD	NON-TREATED CONTROL
G1	**13.68± 2.07 Ab**	**8.70±2.71 Ba**
G2	**15.88± 2.84 Aa**	**8.08± 2.08 Ba**

Means followed by different letters (capital letters horizontally and different non-capital letter vertically) represent statistically significant difference by ANOVA / Tukey test ($p < 0.05$).

Table 4 - Mean ± SD of number of TRAP-positive cells/mm2 in a 1000 µm zone on fenestration defects

GROUPS	TREATED WITH EMD	NON-TREATED CONTROL
G1	**25.95± 1.94 Aa**	**16.97± 1.09 Ba**
G2	**17.01± 3.41 Ab**	**9.52± 1.94 Bb**

Means followed by different letters (capital letters horizontally and different non-capital letter vertically) represent statistically significant difference by ANOVA / Tukey test ($p < 0.05$).

FIGURE LEGENDS

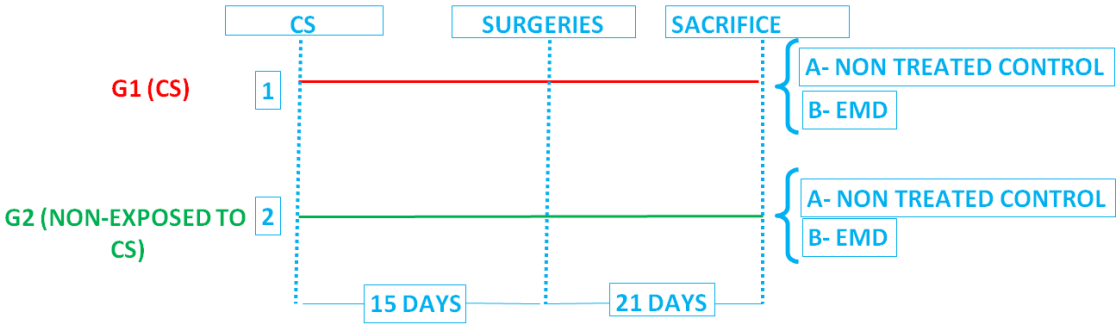

Figure 1: Schematic illustration of the experimental design.

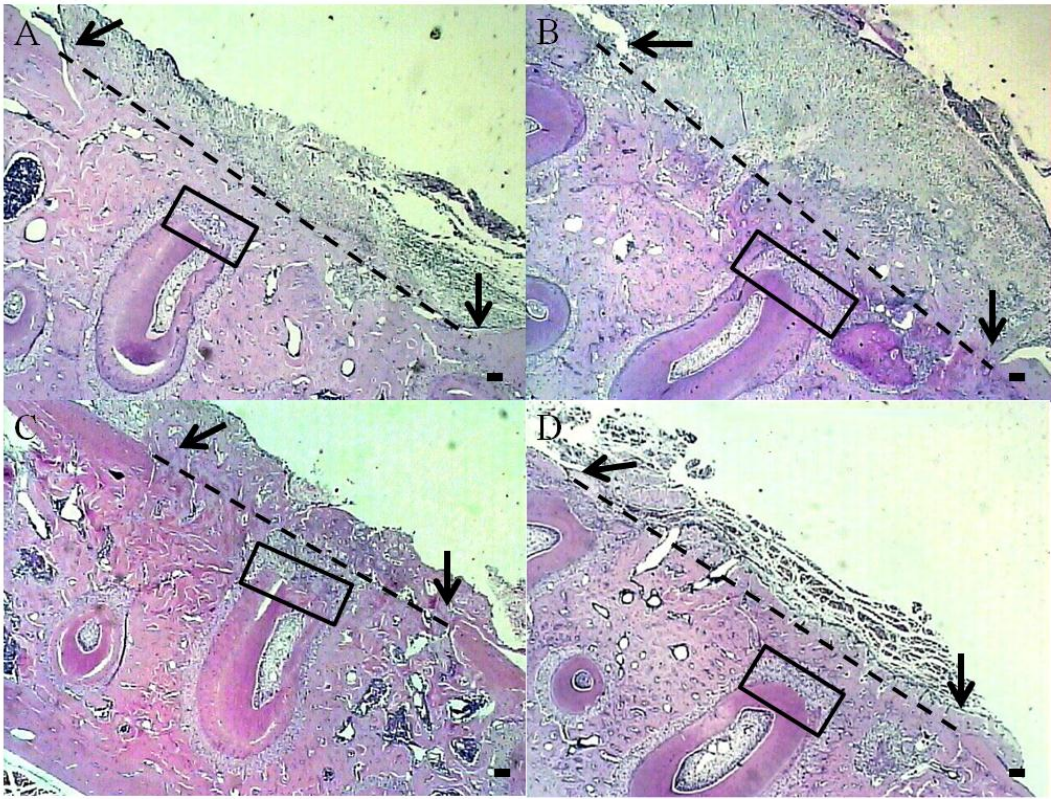

Figure 2: Histological illustration of periodontal healing 21 days after the surgical creation of the fenestration-type defects (H&E, original magnification x 5). A) G1 (CS), non-treated control. B) G1 (CS), treated with EMD. Bar = 1,000 µm. C) G2 (Non-exposed to CS), non-treated control. D) G2 (Non-exposed to CS), treated with EMD. Bar = 1,000 µm. Lines correspond to the original extension of the defects. These lines were drawn using the external edges (arrows indicate edges) of the defect as a reference. Rectangles indicate root instrumented areas.

CONCLUSÃO

Dentro dos limites desse estudo, pode-se concluir que:

i) o diabetes mellitus pode gerar um efeito negativo sobre a densidade óssea. As proteínas derivadas da matriz do esmalte podem aumentar o preenchimento do defeito na presença do diabetes mellitus ou em condições normais. No entanto, EMD não aumenta significativamente a formação de novo cemento em animais diabéticos;

ii) o estresse crônico produz um efeito negativo significativo sobre a densidade óssea. As proteínas derivadas da matriz do esmalte aumentam o preenchimento do defeito na presença do estresse crônico e aumentam a densidade óssea e formação de novo cemento na presença e na ausência do estresse crônico.

iii) o diabetes mellitus parece ter efeito mais severo sobre os tecidos periodontais, quando comparado com o estresse crônico.

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