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Green tea and EGCG effects on periodontal disease in diabetic rats. Microtomographic and histologic analyses

Efeito do chá-verde e EGCG na doença periodontal em ratos diabéticos. Análise microtomográfica e histológica

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Dedico este trabalho aos meus pais, Daisy e Sebastião.
Meu irmão Guilherme e meu esposo D'Alessandro

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Fernando Pessoa

ABSTRACT

Green tea and EGCG effects on periodontal disease in diabetic rats. Microtomographic and histologic analyses

Aim: Currently, there is a growing concern among the general population regarding the use of natural products. Many of the ways by which green tea and its polyphenols work have yet to be elucidated. Thus, the objective of this study was to verify the known effects of green tea as an antioxidant, modulator of vascularization during the progression of spontaneous periodontitis in type 1 diabetic rats (T1D). Also, to verify if daily administration of EGCG attenuates bone loss. Alveolar in diabetic rats with periodontal disease induced by silk thread ligation. Material and methods: In article 1, normoglycemic (NG) and T1D Wistar rats were divided into two control groups, which received water (NG-W; n=25 and T1D-W; n=25) and two experimental groups which received green tea (NG-GT; n=25 and T1D-GT; n = 25). Periodontal structures were evaluated by microtomographic and histological analysis. The number of cells imunolabeled for VEGF (NcVEGF +/mm^2) and CD31 (NcCD31 + / mm^2) as well as the microvessel density (MVD) in the periodontal ligament (PDL) were evaluated. In article 2, 120 Wistar rats were divided into: water treatment (NG-WT, n =20 and T1D-WT n =20), daily treatment with EGCG (NG-EGCG, n =20 and T1D-EGCG, n =20) daily saline treatment (NG-Sham, n =20 and T1D-Sham, n =20). Periodontitis was induced by a ligature placed around the right lower first molar 7 days after initiation of treatment. After 0, 7, 14 and 21 days, the scores of degrees of periodontal disease, PBL and BV / TV were analyzed. Results: In article 1, there was a severe degree of periodontitis with greater reduction in bone volume and periodontal bone level. In T1D-GT, green tea maintained MVD, NcCD31 + / mm2 and NcVEGF + / mm^2 in LDP, being similar to normoglycemic groups. Clinically, in T1D-GT rats, green tea reduced dental plaque accumulation and the degree of periodontitis when compared to T1D-W. In article 2, gradual increase of total PBL was observed in all experimental groups up to 14 days. At 21 days, total PBL of T1D-WT and T1D-Sham increased by an average of 132%, while in NG-WT, NG-Sham, NG-EGCG and T1D-EGCG remained similar. Between 14 and 21 days, a significant increase ($p > 0.01$) of interradicular BV / VT was observed in the normoglycemic and T1D-EGCG groups. T1D-EGCG PD scores did not show statistical differences when compared to NG groups. Conclusion:

Daily consumption of green tea has a therapeutic effect on diabetic vascular disorder in the PDL and the progression of periodontitis in the long-term of hyperglycaemia in T1D rats, whereas daily consumption of EGCG has therapeutic effect on periodontal disease in hyperglycemic condition, reducing then the degree and severity of the disease.

Key-words: Antioxidants, Polyphenol Oxidase, Diabetes Mellitus, Catechin, Periodontal Disease, Histological Techniques, X-Ray Microtomography

RESUMO

Efeito do chá-verde e EGCG na doença periodontal em ratos diabéticos. Análise microtomográfica e histológica

Objetivo: Atualmente, existe uma grande preocupação da população em geral no uso de produtos de origem natural. Muitas das maneiras pelas quais o chá-verde e seus polifenóis atuam ainda precisam ser elucidadas. Assim, o objetivo deste trabalho foi verificar os efeitos conhecidos do chá-verde como antioxidante e modulador da vascularização durante a progressão da periodontite espontânea em ratos diabéticos tipo 1 (T1D) a longo prazo e verificar se a administração diária de EGCG atenua a perda óssea alveolar em ratos diabéticos com doença periodontal induzida por ligadura com fio de seda. Material e métodos: No artigo 1, ratos *Wistar* normoglicêmicos (GN) e T1D foram divididos em dois grupos controle, que receberam água (GN-W; n = 25 e T1D-W; n = 25) e dois grupos experimentais que receberam chá-verde (NG-GT; n = 25 e T1D-GT; n = 25). As estruturas periodontais foram avaliadas por análises microtomográficas e histológicas. Foram avaliados o número de células imunmarcadas para VEGF (NcVEGF + / mm2) e CD31 (NcCD31 + / mm2), bem como a densidade de microvasos (MVD) no ligamento periodontal (PDL). No artigo 2, 120 ratos Wistar foram divididos em: tratamento com água (NG-WT, n = 20 e T1D-WT n = 20), tratamento diário com EGCG (NG-EGCG, n = 20 e T1D-EGCG, n = 20) tratamento diário com solução salina (NG-Sham, n = 20 e T1D-Sham, n = 20). A periodontite foi induzida por ligadura ao redor do primeiro molar inferior direito 7 dias após o início do tratamento. Após 0, 7, 14 e 21 dias, foram analisados os escores do grau de doença periodontal, PBL e BV / TV. Resultados: No artigo 1, observou-se grau severo de periodontite com maior redução no volume ósseo e no nível ósseo periodontal. No T1D-GT, o chá verde manteve o MVD, NcCD31+/mm^2 e NcVEGF+/mm^2 no PDL, sendo semelhante aos grupos normoglicêmicos. Clinicamente, em ratos T1D-GT, o chá verde reduziu o acúmulo de placa dentária e o grau de periodontite quando comparado ao T1D-W. No artigo 2, aumento gradual do PBL total foi observado em todos os grupos experimentais até 14 dias. Aos 21 dias, o PBL total de T1D-WT e T1D-Sham aumentou em média 132%, enquanto no NG-WT, NG-Sham, NG-EGCG e T1D-EGCG permaneceram semelhantes. Entre 14 e 21 dias, foi observado um aumento significativo (p> 0,01)

da BV/TV interradicular nos grupos normoglicêmicos e T1D-EGCG. Os escores de DP no T1D-EGCG não apresentaram diferenças estatísticas quando comparados aos grupos NG. Conclusão: O consumo diário de chá verde tem um efeito terapêutico no distúrbio vascular diabético nas PDL e na progressão da periodontite na hiperglicemia a longo prazo em ratos T1D, enquanto o consumo diário de EGCG tem efeito terapêutico na doença periodontal na condição hiperglicêmica, reduzindo o grau de gravidade da doença.

Palavras-chave: Antioxidantes, Polifenol Oxidase, Diabetes Mellitus, Catequinas, Doença Periodontal, Técnicas Histológicas, Microtomografia

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1 INTRODUCTION

1 INTRODUCTION

Diabetes miellitus (DM) is a disease that occurs because the pancreas no longer produces enough insulin or the body cannot effectively use the insulin it produces. Hyperglycaemia or increased blood sugar is the most common effect of decompensated diabetes. Diabetes is one of the chronic diseases that has a greatest impact on health spending because, if poorly controlled, it brings severe macro and microvascular complications that burden health services. World health organization (WHO) data have pointed a large increase of the prevalence of this disease worldwide. In this context, Brazil appear as the 8th country with the highest prevalence of the disease (WHO, 2019).

DM damages various organs then cause systemic complications, including periodontal disease. These changes are usually present when there is poor metabolic control. The prevalence of periodontal disease in diabetics is much higher than in the general population. Accordingly, it is believed that 4% of adults receiving oral treatment are diabetic (Orso and Pagnoncelli, 2002; Sousa *et al.*, 2003; Negrato and Tarzia, 2010).

The mechanisms by which hyperglycaemia influences the periodontium are similar, in many aspects, to the pathophysiology of various classic diabetic complications such as nephropathy, retinopathy and cardiomyopathy (Mealey and Oates, 2006). Chronic hyperglycaemia increases glycation proteins and lipids promoting inflammatory response in tissues, microvascular damage in the periodontium, changes in the composition of crevicular fluid and host bacterial flora of the gingiva as well as unbalanced healing response in the periodontium. The blood vessels are essential for successful healing or progression of inflammatory process (Lalla and Papapanou, 2011; Vasconcelos *et al.*, 2016).

The involvement of blood vessels in the degree of inflammation is due to the ability of new vessels to carry inflammatory cells to the lesion and to supply oxygen and nutrients to inflamed tissues (Johnson *et al.*, 1999). From various cytokines and growth factors involved in angiogenesis, the most potent agent is vascular endothelial growth factor(VEGF) (Connolly, 1991; Ferrara *et al.*, 1992; Dvorak *et al.*,

1995; Lantieri *et al.*, 1998; Becit *et al.*, 2001; Hayashibara *et al.*, 2001). VEGF potentially increases vascular permeability, stimulates endothelial cell proliferation, induces proteolytic enzyme expression and endothelial cell, monocyte and osteoblast migration, all essential for angiogenesis (Connolly, 1991; Ferrara *et al.*, 1992; Dvorak *et al.*, 1995; Nakagawa *et al.*, 2000; Sakuta *et al.*, 2001). According to (Artese *et al.*, 2010), VEGF is an important factor for the pathogenesis of aggressiveness and chronic form of periodontitis. The concept that specific microorganisms act as etiological agents of periodontal disease resulting in bone loss and dental insertion is well established and accepted in the literature (Haffajee and Socransky, 1994).

In this context, much of the destruction of periodontal tissues is due to the host response dysfunction that exacerbates the expression and or activation of intracellular signaling molecules such as polymorphonuclear leukocytes, altering collagen metabolism and vascular permeability, reducing viability and differentiation of cells in the periodontium, and altering microflora (Mealey, 1999; Lalla *et al.*, 2001; Hudson *et al.*, 2003). Therefore, diabetes may induce periodontal disease during dysregulation of the immune and inflammatory response against commensals of the periopathogenic microbiota (Garlet *et al.*, 2013) and (Lamster and Novak, 1992). This process ends promoting the expansion of the vascular network (Lucarini *et al.*, 2009), aggravates periodontal disease by VEGF-mediated dynamic tyrosine phosphorylation of cell junction proteins such as VE-cadherin and PECAM-1/CD31, an important modulatory step for endothelial cell adhesion and migration (Esser *et al.*, 1998).

Most of the tissue and cellular changes that occur in the hyperglycemic state are due to irreversible formation Advanced Glycated End Products (AGEs). Through the generation of this radicals, the formation of protein cross-links or interactions with cell receptors, AGEs promote, respectively, oxidative stress, morphofunctional changes and increased expression of inflammatory mediators. In addition, after inflammatory stimulation, such as in periodontal disease, neutrophils, monocytes and macrophages produce myeloperoxidase and the enzyme NADPH oxidase, which induce the formation of AGEs by amino acid oxidation. Locally generated AGEs interact with RAGEs (cell surface receptors) (Schmidt *et al.*, 1992), initiating and propagating a RAGE-dependent inflammatory response.

Oxidative stress, defined as an imbalance between prooxidant and antioxidant systems, is been proposed as a single unifying mechanism linking the various biochemical pathways triggered by hyperglycaemia (Nishikawa *et al.*, 2000; Brownlee, 2005); this highlights the potential therapeutic role of antioxidants in people with poor control of diabetes to prevent or delay the development of vascular complications.

Increased RAGE expression and proinflammatory cytokines has been reported in experimental models of diabetes-associated periodontal disease (Chang *et al.*, 2012; Claudino *et al.*, 2012; Chang *et al.*, 2013) and in diabetic individuals with periodontitis (Katz *et al.*, 2005; Abbass *et al.*, 2012; Yu *et al.*, 2012). These results demonstrated that the AGE-RAGE interaction lead to an exacerbated inflammatory response and periodontal tissue destruction in diabetes.

Among therapeutic options for DM (Negri, 2005), green tea is one of the most consumed beverages in the world. It is obtained from the leaves of *Camellia sinensis* that belongs to the *Theacea* Family, genus *Camellia* and the specie *sinensis.* It is possible to obtain various types of tea, the most widely used are green tea from dried leaves, and the black tea obtained by infusion of the processed leaves (Trevisanato and Kim, 2000; Matsubara *et al.*, 2006).

There is a growing interest in the therapeutic effects of natural antioxidant substances such as polyphenols, which are abundant in plant derived foods, especially fruits, seeds and leaves, as they can strengthen the body's defense against various diseases and help to maintain a healthy oral environment (Petti and Scully, 2009; Venkateswara *et al.*, 2011; Lolayekar and Shanbhag, 2012).

Given the multifactorial etiology of periodontitis, our research group previously proposed the use of green tea. Then, in a first phase study, it was observed that green tea intake reduces expression of the pro-inflammatory cytokine TNF-α and the osteoclastogenic mediator RANKL to normal levels while increasing expression of the anti-inflammatory cytokine IL-10, the osteogenesis-related factor RUNX-2 and the anti-osteoclastogenic factor OPG. (Gennaro *et al.*, 2015).

Following this research line we subsequently published the Article 1 (Catanzaro *et al.*, 2018) in which we approached the daily green tea consumption as

a therapeutic effect on the diabetic vascular disorder in the periodontal ligament and the progression of periodontitis in long-term hyperglycaemia in T1D rats.

There we realized that the beneficial effects of green tea on periodontal disease in diabetic rats were very clear, then we decided to study EGCG, the most active and abundant component of green tea approached in the Article 2, still in elaboration. From four catechins found in green tea, Epigallocatechin-3-gallate (EGCG) is the more abundant, accounting about 10% of the whole composition. Studies show that one cup of green tea (equivalent to 2.5 grams of green tea leaves / 200 ml of water) contains 90 mg EGCG (Venkateswara *et al.*, 2011). The recommended consumption is three to four cups of tea per day, and the average cup of green tea contains about 50-150 mg of polyphenols.

Recent studies have shown that polyphenols in green tea exhibit anti-tumor activity and that might be one of the possible mechanisms of action. That is through modulation of the angiogenesis signaling cascade (Wahl *et al.*, 2011). According to some authors (Jung *et al.*, 2001; Masuda *et al.*, 2002; Zhang *et al.*, 2006; Zhu *et al.*, 2007; Tang *et al.*, 2008; Ohga *et al.*, 2009; Shimizu *et al.*, 2010; Yang and Wang, 2010; Mizushina *et al.*, 2011; Singh *et al.*, 2011; Yang and Wang, 2011; Yang *et al.*, 2011; Thakur *et al.*, 2012; Tudoran *et al.*, 2012), epilocatechins present in green tea may inhibit the activation axis of VEGF and its receptors by suppressing HIF-α and other growth factors.

In a study published in Journal of Periodontology, (Kushiyama *et al.*, 2009) analyzed the periodontal health of 940 men and found that those who drank green tea regularly had better periodontal health than those who consumed less of it. The researchers also noted that for each cup of green tea consumed per day, there was a decrease in all three indicators of periodontal disease: periodontal pocket depth (PD), loss of clinical gingival tissue insertion (CAL), and bleeding on probing (BOP), which means a lower predisposition to periodontal disease in individuals who regularly drink green tea. According to the authors, the ability of green tea to reduce the symptoms of periodontal disease is due to the presence of catechins, a potent antioxidant.

This molecule acts by interacting in various ways with biomolecules such as proteins, lipids and nucleic acids (Nozaki *et al.*, 2009). EGCG not only binds enzymes that act on DNA transcription activating molecules, but is also capable of

binding directly to DNA and RNA (Balasubramanian and Eckert, 2004), protecting against free radical damage, ionization, ultraviolet radiation and DNA methylation that can induce the cancer cell (Suganuma *et al.*, 1996). Several studies have also shown that EGCG suppresses LPS-induced bone resorption by inhibiting IL-1β production or directly by inhibiting osteoclastogenesis (Yun *et al.*, 2004; Rogers *et al.*, 2005; Yun *et al.*, 2007). In addition, EGCG inhibits RANKL-induced osteoclast differentiation via suppression of NF-kβ transcriptional activity (Lee *et al.*, 2009).

According Shen *et al., (*2013) (Shen *et al.*, 2013)[66](SHEN; KWUN; WANG; MO *et al.*, 2013)(SHEN; KWUN; WANG; MO *et al.*, 2013) as the antioxidant and anti-inflammatory properties of green tea, catechins are capable of promoting osteoblastogenesis, suppressing osteoclastogenesis and stimulating differentiation of mesenchymal stem cells into osteoblasts rather than via kinase signaling pathways (ERK). In the studies by LEE *et al.*, (2010) EGCG prevented osteoclast differentiation in bone marrow cell coculture with primary osteoblasts after induction with IL1, TNF-α and Vitamin D3 + PGE2, and TRAP-positive multinucleated cells decreased in a dose-dependent manner with EGCG treatment (Yun *et al.*, 2004). For the authors, EGCG has an anti-osteoclastogenic effect, being suggested as a treatment option for various bone pathologies with excessive osteoclast formation and bone destruction. It is noteworthy that most studies evaluating the effect of EGCG on osteoclastogenesis are recent and have been performed *in vitro.* Regarding periodontal disease we have the work of (Cho *et al.*, 2013) who evaluated in rats the therapeutic effect of orally administered EGCG after onset of periodontal disease induced during 7 days of ligature. They found that systemic administration of EGCG had a therapeutic effect on periodontal disease, reducing the number of osteoclasts due to decreased expression of inflammatory cytokines such as TNF and IL-6.

In this study, we aimed to evaluate the potential effects of green tea and EGCG in the periodontal vascular disorder and periodontitis progression as a consequence of over time hyperglycaemia in T1D rats. Thus, X- ray microtomographic, histopathologic and immunohistochemical analyses were performed to verify the VEGF and CD31 expression, also microvessel density (MVD) and structural integrity of periodontal tissues in normoglycaemic and hyperglycaemic rats with and without green tea intake.

2 ARTICLES

2 ARTICLES

The articles presented in this thesis were written according to the instructions and guidelines for article submission of the corresponding journals.

- ARTICLE 1 – Green tea prevents vascular disturbs and attenuates periodontal breakdown in long-term hyperglycaemia in T1D rats. Journal of Clinical Periodontology. (Accepted)
- ARTICLE 2 – Epigallocatechin gallate of green tea attenuates progression of periodontitis induced by ligature in diabetic rats. Journal of Clinical Periodontology. (In preparation)

2.1 ARTICLE 1 – Green tea prevents vascular disturbs and attenuates periodontal breakdown in long-term hyperglycaemia in T1D rats*. (Appendix 1 and Annex 1).

ABSTRACT

Aim: The effects of green tea on the modulation of vascularization during the progression of spontaneous periodontitis in long-term hyperglycaemia in streptozotocin-induced type 1 diabetic (T1D) rats were evaluated. **Materials and Methods:** Wistar rats normoglycaemic (NG) and T1D were divided into two control groups, which received water (NG-W and T1D-W) and two experimental groups that received green tea (NG-GT and T1D-GT). Periodontal structures were evaluated by microtomographic and histological analyses. Number of immunostained cells for VEGF (NcVEGF+/ mm^2) and CD31 (NcCD31+/ mm^2), as well microvessel density (MVD) in the periodontal ligament (PDL) were evaluated. **Results:** Long-term hyperglycaemia in T1D-W rats induced vascular alterations in PDL with a reduction of 36% in MVD, a decrease of 33% in NcCD31+/ mm^2 and an increase of 53% in NcVEGF+/mm^2. Concomitantly, a severe degree of periodontitis with higher reduction in bone volume and periodontal bone level was observed. In T1D-GT, green tea maintained the MVD, NcCD31+/mm^2 and NcVEGF+/mm^2 in the PDL similar to normoglycaemic groups. Clinically, in T1D-GT rats, green tea reduced dental plaque accumulation and the degree of periodontitis when compared to T1D-W. **Conclusion:** Daily green tea consumption has a therapeutic effect on the diabetic vascular disorder in PDL and the progression of periodontitis in long-term hyperglycaemia in T1D rats.

Keywords: antioxidants, CD31, diabetes mellitus, green tea, periodontal diseases, VEGF, X-ray Microtomography.

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2.1 ARTICLE 1 – **"**This is the peer reviewed version of the following article: ***[Green tea prevents vascular disturbs and attenuates periodontal breakdown in long-term hyperglycaemia in T1D rats],*** which has been published in final form at https://onlinelibrary.wiley.com/doi/pdf/10.1111/jcpe.12883. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions." (Appendix 1 and Annex 1).

Green tea prevents vascular disturbs and attenuates periodontal breakdown in long-term hyperglycaemia in T1D rats

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CLINICAL RELEVANCE

Scientific rationale for the study: Studies showed a correlation between diabetes and periodontitis. Long-term of poor glycemic control in diabetic patients leads to systemic microvascular alterations who is also considered a risk factor for periodontitis. The use of natural products rich in antioxidants like green tea have a possible strategy in the treatment of vascular diseases and periodontitis.

Principal findings: Green tea improves glycemic control, tissue vascularization and decreases the accumulation of dental plaques and periodontal tissue loss in long-term of hyperglycaemia in T1D rat.

Practical implications: Green tea can be used as a possible therapy adjunct to mechanical oral hygiene procedures in diabetic patients who have a risk of poor glycemic control.

1 INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease characterized by hyperglycaemia resulting from total or partial deficiency in the synthesis and secretion of insulin (Berezin, 2016). Clinical studies showed that the incidence of chronic gingivitis and periodontitis is significantly higher in patients with type 1 diabetes (T1D) than that of the healthy population especially when associated with a long-term poor glycaemic control (Llambes, Silvestre, Hernandez-Mijares, Guiha, & Caffesse, 2005; Seppala, Sorsa, & Ainamo, 1997). The observation that hyperglycaemia by itself, in the absence of additional inflammatory signals, promotes a proinflammatory environment indicates that diabetes is an independent risk factor for the development of periodontal disease (Graves & Cochran, 2003; Hickey & Kubes, 2009; Medzhitov, 2008). The mechanisms by which hyperglycaemia influences the periodontium are similar in many respects to the pathophysiology of various classic diabetic complications such as nephropathy, retinopathy and cardiomyopathy (Mealey & Oates, 2006). Chronic hyperglycaemia increases glycation proteins and lipids that promote inflammatory response in tissues, microvascular damage in the periodontium, changes in the composition of crevicular fluid and host bacterial flora of the gingiva and unbalanced healing response in the

periodontium. The blood vessels are essential for successful healing or progression of inflammatory process (Lalla & Papapanou, 2011; Vasconcelos et al., 2016).

Chronic hyperglycaemia is recognized as the major responsible factor for the development of all types of diabetes-specific microvascular disorders. Intracellular hyperglycaemia causes abnormalities in the blood flow, increased rate of apoptosis and hyper-permeability by alteration of gene expression or protein function (Kitada, Zhang, Mima, & King, 2010). The pathogenetic sequence of diabetic microangiopathy is interrelated with biochemical abnormalities associated with hyperglycaemia that can lead to altered expression or action of several factors, such as insulin, PDGF, VEGF or APC, which are physiologically important for keeping the homoeostasis of vasculatures. Among them, VEGF is the most potent and primary endothelial-specific angiogenic growth factor, both in physiological and pathological conditions as tumour progression (Shibuya, 2008) and diabetic retinopathy and nephropathy (Hanes & Krishna, 2010). In the periodontal tissues, the VEGF acts as a potent inflammatory agent in periodontitis, especially when aggravated by diabetes (Aspriello et al., 2009). This factor has been related to the initiation and progression of gingivitis to periodontitis, promoting the expansion of the vascular network (Lucarini et al., 2009). VEGF-mediated dynamic tyrosine phosphorylation of cell junction proteins such as VE-cadherin and PECAM-1/CD31 is an important modulatory step for endothelial cell adhesion and migration (Esser, Lampugnani, Corada, Dejana, & Risau, 1998).

Oxidative stress, which is defined as an imbalance between prooxidant and antioxidant systems, has been proposed as a single unifying mechanism linking the various biochemical pathways triggered by hyperglycaemia (Brownlee, 2005; Nishikawa, Edelstein, & Brownlee, 2000); it highlights the potential therapeutic role of antioxidants in people with poor control of diabetes to prevent or delay the development of vascular complications. Green tea (GT) is a therapeutic option derived from medicinal plant *Camellia sinensis* and emerges scientifically as good option for inflammatory diseases treatment (Khan & Mukhtar, 2013). The polyphenols present in *C. sinensis* have shown important biological benefits in the body in certain disorders, such as diabetes, cancer, heart diseases, viral infections, inflammation, caries and periodontal diseases (Ramasamy, 2015). In previous work performed in our laboratory (Gennaro et al., 2015), it was observed that daily ingestion of GT

reduced the expression of the pro-inflammatory cytokines TNF-α and the osteoclastogenic mediator RANKL in diabetic rats.

Thus, in this study, we aimed to evaluate the potential effects of green tea in the periodontal vascular disorder and periodontitis progression as a consequence of over time hyperglycaemia in T1D rats. Thus, X- ray microtomographic, histopathologic and immunohistochemical analyses were performed to verify the VEGF and CD31 expression, also microvessel density (MVD) and structural integrity of periodontal tissues in normoglycaemic and hyperglycaemic rats with and without green tea intake.

2 MATERIAL AND METHODS

2.1 Animals

Animal experimental procedures were approved by the Ethics Committee of Bauru School of Dentistry, University of São Paulo (Protocol: CEEPA-008/2012). A hundred male Wistar rats (*Rattus novergicus*) at 60 days of age and weighing around 250 g were used. They were maintained in home cages under conditions of controlled temperature and humidity with 12:12 hr light/dark cycle and free access to food and water.

2.2 Experimental design

Experimental design (Figure 1) was performed as previously described by our research group (Gennaro et al., 2015). Briefly, T1D rats were induced by a single intraperitoneal injection of 47 mg/kg of streptozotocin (Sigma, St. Louis, MO, USA). Seven days after STZ administration, T1D (n = 50) and normoglycaemic (NG, n = 50) rats were subdivided into four groups according to the treatment: T1D-GT (n = 25) and NG-GT (n = 25) receiving only green tea ad libitum (the preparation of the green tea was performed according to the method used by Gennaro et al., 2015), then T1D-W (n = 25) and NG-W (n = 25) receiving distilled water ad libitum.

2.3 Hemimandible collection and macroscopic evaluation of tooth decay

After 0, 15, 30, 60 and 90 days of treatment, weight and fasting glucose levels (FGL) were measured; then, the animals were sacrificed by an overdose of ketamine and xylazine hydrochloride (Vetbrands Brazil Limited, Jacareí, SP). Hemimandibles were removed and fixed in 10% phosphate-buffered formalin solution at pH 7.2 for 1 week. Macroscopic images of the region molars from each hemimandible are obtained using a digital microscope and capture software (Dino-Lite-USB Digital Microscope and DinoCapture 2.0, Taiwan, China) for qualitative assessment to of oral health and tooth decay by scores as indicated in Table 1.

2.4 Micro-CT assessment and determination of periodontal bone level

All hemimandibles were scanned in a microtomography scanner Skyscan 1176 (Bruker, Kontich, Belgium) at 50 kV and 800 μA with a resolution of 14 μm per pixel and rotation of 180 degrees with steps of 0.8°. The images generated were reconstructed and reoriented spatially according to the literature (Chang et al., 2013), and the periodontal bone level (PBL) was determined by mean of the sixteen distances obtained from the CEJ to the alveolar bone crest as a de-scribed in Figure 1b.

2.5 Histological processing and histopathological analysis of periodontitis

The hemimandibles were decalcified with 4.13% ethylenediamine tetraacetic acid (EDTA) 7.2 pH for 8 weeks and processed histologically for embedding into polymer-enriched paraffin (Histosec™ Merck KGaA - Darmstadt, Germany). Longitudinal 4-μm- thick serial sections were obtained and placed on silane-coated glass slides (Dako-Japan Co., Ltd, Kyoto, Japan).

Five sections stained with haematoxylin and eosin per hemimandible showing the central portion of the coronal and radicular pulp chamber of the first molar were selected. The analyses were by a single examiner using an image capture in AxioCam HRc sys-tem and AxioVision software (Carl Zeiss, Gottingen, Germany). For each image, the periodontitis is analysed by scores as indicated in Table 1.

2.6 Immunohistochemical procedures for determination NcVEGF+ and NcCD31+ per mm2 of PDL and microvascular density (MVD)

Three sections were stained by immunohistochemical technique. The endogenous peroxidase activity was blocked by 3% hydrogen peroxide and antigen retrieval in citrate buffer at 95°C. After normal serum blocking, the sections were incubated with rabbit polyclonal antibody against CD31 (ABCAM® Cambridge, United Kingdom) and VEGF (Santa Cruz Biotechnology® Inc., Dallas, Texas) for 1 hr, while the negative controls were incubated only with antibody diluent. Subsequently, anti-rabbit secondary antibody (N- Histofine® Simple Stai™ Rat MAX PO, Nichirei Biosciences INC, Co., Japan) was applied for 30 min and the reaction visualized by diaminobenzidin DAB (Nichirei Co., Japan). For each hemimandibles, 42 histological fields (14 fields per section) in the periodontal ligament (PDL) of the first molar were captured using a digital camera AxioCam HRc attached to a microscope Axioscop 2 (Carl Zeiss, Gottingen, Germany) with a 40X oil immersion objective. Using AxioVision software (Carl Zeiss, Gottingen, Germany), the VEGF- and CD31-positive cells were counted and the number of immunolabelled cells per mm2of tissue calculated. For MVD, the area of vessels immunostained against CD31 (Ava) and total area exanimated (AT) were determined, and MVD was calculated by ratio MVD = (Ava/AT) × 100.

2.7 Statistical analysis

The data were expressed as mean ± standard error of the mean. All tests were performed with Statistica software 10.0 (StatSoft Inc., Tulsa, OK, USA). First, the normality of distribution of data and homogeneity of variance were evaluated by Hartley, Cochran and Bartlett tests. One-way ANOVA and Tukey's post hoc test were applied to test the influence of time/period on the weight, FGL, PBL, NcVEGF+ and MVD in each experimental group and differences among groups in each period. Similarly, the histopathological scores were compared between subgroups using the Kruskal–Wallis tests. The level of significance for all cases was established in $p < .05$.

3 RESULTS

3.1 Laboratorial data showed that daily consumption of green tea had a moderate antihyperglycaemic effect

The evolution of weight, tea/water intake and FGL remained within average levels expected for healthy rats in normoglycaemic groups. The body weight increased 43.7% until 90 days (415.3 ± 22.4 g), while daily tea/water intake (22.3 ± 3.6 ml/day) and FGL (85.7 ± 6.2 mg/dl) maintained stable throughout the experiment. In the T1D animals, the tea/water intake (96.1 ± 26.8 ml/day) and glycaemic index (320.7 ± 34.2 mg/dl) were significantly higher when compared with NG rats in all experimental periods, while body weight was 36% smaller (247.1 ± 9.9 g). However, significant improvements in the clinical conditions were observed in the animals that consumed green tea compared to those which consumed water such as lower hydric in-take (74.1 ml/day versus 118 ml/day), glycaemic level (284.4 mg/dl versus 357.2 mg/dl) and diuresis associated with higher body weight (243 mg versus 219 mg). In T1D-GT rats, a moderate reduction in initial glycaemic levels (307.1 mg/dl at 0 day and 195.0 mg/dl) at 15 days was observed.

3.2 Macroscopic and microtomographic analysis showed smaller dental plaque accumulation and bone height loss in T1D rats treated with green tea

Macroscopic (Figure 2) and microtomographic (Figure 3) features of molars showed absence of dental plaque accumulation and preservation of periodontal and dental structures along of the experimental periods in normoglycemic groups. In T1D-W rats, occurred large supra and sub-gingival plaque formation principally on lingual tooth surface until 30 days (Figure 2) accompanied by increase of 93% of PBL (0.45μm to 0.86μm, Figure 4A). After 60 days, higher bone height loss and extensive caries lesions with pulp exposure were evident in all animals of T1D-W (Figures 2 and 3). In T1D-GT) small plaque accumulation, mild and moderate bone height loss and presence of initial/moderate caries lesion was observed only after 60 days (compare the images of figures 2 and 3). At 90 days, the PBL (Figure 4A) in the T1D-GT (0.93 ± 0.11μm) was 0.31% smaller compared to T1D-W (1.34 ± 0.21mm), but 46% higher in relation to normoglycemic groups (0.63 ± 0.15mm). In 2D and 3D microtomographic images at 90 days (Figure 4B) show preservation of bone crest

level in normoglicemic rats compared to diabetic rats and smaller bone height loss in T1D-GT than T1D-W.

3.3 Histopathological analysis showed that green delays the evolution of periodontal disease and tooth decay in T1D rats

The histological features of M1 (Figure 5) pertaining to NG-W and NG-GT groups between 0 and 30 days showed integrity in the periodontal and dental tissues, being attributed a score “0” or health (Figures 5B and C). Between 60 and 90 days, a score of “1” or gingivitis was attributed to 4/10 of M1 evaluated. In the T1D rats, a progressive loss of periodontal and dental tissues was verified after 30 days being more severe in T1D-W compared to T1D-GT (compare the Figure 5A and graphic evolution of Figures 5B and 5C). In T1D-W intense inflammatory process in periodontal tissues, severe gingival recession, higher bone loss, deep caries principally in the gingival third of lingual and mesial surfaces of tooth and pulpitis/necrosis were observed at 60 to 90 days. At 90 days endpoint, a score of 3 (moderate) to 4 (advanced) for periodontitis and 2 (dentin decay) to 4 (abscess formation) for tooth decay were attributed. In T1D-GT only early (score “1”) and moderate (score “2”) periodontitis and enamel decay (score “1”) to dentin decay (score “2”) were observed.

3.4 Immunohistochemical evaluations showed that green tea consumption inhibits or delays diabetic-microvascular alterations in PDL of T1D-W

Immunolabeling for VEGF (Figure 6A) was observed in osteoblasts in bone formation areas, osteoclasts in bone resorption areas, fibroblasts and endothelial cells along the PDL. CD31/PECAM-1 was primarily concentrated in endothelial cells and leukocytes (Figure 6C). The NcVEGF+ (Figure 6B) and NcD31+ (Figure 6D) and MDV (Figure 6E) were similar in NG groups and T1D-GT during all periods (14.0 ± 0.64 x 102cells/mm2; 17.2 ± 1.31 x 102cells/mm2 and 5.3 ± 0.23%, respectively) except by a peak of MVD at 15 days (6.9 ± 0.43%) in NG-GT and a peak at 30 days in T1D-GT (6.5 ± 0.46%). In T1D-W, the NcVEGF+ was smaller than NG groups until 30 days (11.9 ± 0.68 x 102 cells/mm2 vs. 14.7 ± 1.41 x 102 cells/mm2). After this period, the NcVEGF+ increased 13.6% (17.5 ± 1.28 x 102 cells/mm2) accompanied by a decrease of 34% in the NcCD31+ (10.6 ± 1.5 x 102 cells/mm2) and 36% in MVD (3.2 ± 0.41 x 102 cells/mm2).

4 DISCUSSION

One characteristic complication in long-term poor glycemic control is the appearance of vascular abnormalities linked to advanced periodontal disease with severe loss of alveolar bone (Holtfreter et al., 2013). This study highlights the effects of green tea on the relationship between diabetic vascular complications and periodontal disease progression.

The microtomographic and histomorphometric results showed that the dental and periodontal tissues remained healthy in the NG groups along the experimentation period except by a mild gingival inflammation at 90 days endpoint. Regarding to diabetic groups, the animals of T1D-W group showed a large supra and sub-gingival dental plaque deposit along the cervical margin, preferentially on the lingual side of molars and periodontal pockets at 30 days. Subsequently, the gingivitis progresses into advanced periodontitis with extensive vertical bone loss exposing the root apex and leading to tooth mobility at 90 days. Concomitantly, evolution of deep caries principally of class V with pulp exposure also observed. It was clear that the dental plaque that forms and remains on tooth surfaces is the one main factor in spontaneous periodontal disease and caries in hyperglycemic T1D rats. Clinical trial studies show major incidence of dental plaque, chronic gingivitis and periodontitis in T1D patients than that of healthy subjects (Lalla et al., 2006; Orbak, Simsek, Orbak, Kavrut, & Colak, 2008; Salvi et al., 2010). Comparatively, other clinical reports have provided that the hyperglycemic environment increase of the virulence of some pathogens; lower production of interleukins in response to infection; reduced chemotaxis and phagocytic activity, immobilization of polymorphonuclear leukocytes; glycosuria, gastrointestinal and urinary dysmotility (see review) (Schuetz, Castro, & Shapiro, 2011) increasing the susceptibility to infections in diabetic patients. Long-term hyperglycaemia in T1D mice decreased 50% the phagocytic ability of leukocytes reducing the capacity of organism to clear bacterial infections (Pettersson et al., 2011).

Green tea treatment in T1D-GT provided a greater glycemic control, small dental plaque accumulation when compared to T1D-W. The first signs of periodontal disease and dental caries appeared only at 60 days' time-point and the disease evolutions were less intense compared to T1D-W. These results show an inverse

association between green tea intake and periodontal and dental disease, which is in accordance with the clinical results (Kushiyama, Shimazaki, Murakami, & Yamashita, 2009). The favorable effects can be attributed to antioxidant capacity of green tea. In recent study, oral administration of green tea extract (200 mg/Kg) significantly decreased the serum glucose level and serum and hepatic oxidative stress biomarkers and increase de total of oxidants capacity (Haidari, Omidian, Rafiei, Zarei, & Mohamad Shahi, 2013). Clinically, in a 3-month follow-up of a randomized controlled clinical trial in patients with mild to moderate periodontitis, the green tea intake increased 8-fold antioxidant capacity in gingival crevicular fluid improving the clinical parameters of gingival index, plaque index, clinical probing depth, clinical attachment loss, percentage of sites with bleeding on probing of these patients (Chopra, Thomas, Sivaraman, Prasad, & Kamath, 2016). The polyphenols of green tea could have also restored the secretion of antimicrobial peptides, such as beta-defensins, by gingival epithelial cells (Lombardo Bedran, Feghali, Zhao, Palomari Spolidorio, & Grenier, 2014) and/or improved immunomodulatory responses to bacteria by neutrophils (Lalla & Papapanou, 2011) preventing or reducing bacterial biofilm growth and pyogenic bacterial infections.

Vascular response is an essential aspect of an effective immune response to periopathogens (Mendes et al., 2016). In our study, the higher periodontal and dental tissue loss observed in T1D-W rats was associated to vascular alteration in PDL. The evolution of diabetic-microvascular alterations in PDL depended on both the duration and the severity of hyperglycaemia, as observed in other classical-microvascular complications (retinopathy and neuropathy). Thus, at 60 days it was possible to observe the formation of dense and disorganized collagen fibers, reduction of 36% in vessel density/MVD and decrease of 33% in NcCD31+/mm2 associated to increase of 53% in NcVEGF+/mm2 in all PDL of T1D-W rats. Systemically, elevated VEGF expression also has been reported in the serum levels of the T1D adolescents and young adults with microvascular complications compared with both healthy controls and diabetic patients without retinopathy or nephropathy (Chiarelli et al., 2000; Seckin, Ilhan, & Ertugrul, 2006). The excess of VEGF inhibits differentiation of mesenchymal progenitor cells suppressing their further osteogenic maturation (Hu, Besschetnova, & Olsen, 2016; Hu & Olsen, 2016) and stimulates fibroblasts to produce RANKL that indirectly induce osteoclastogenesis (H. R. Kim, Kim, Kim, Cho,

& Lee, 2015). Thus, optimal levels of VEGF were required for coupling of angiogenesis and osteogenesis and control of osteoclastic bone resorption.

In current work, the normoglycemic animals that received green tea the NcVEGF+/mm2 showed a similar pattern to NG-W in all experimental period highlighting that the oral administration of EGCG no effect VEGF expression in the normal tissues. In this respect, the breast cancer in female mice that received 50 mg/kg/day of green tea EGCG-polyphenol showed a significantly inhibition of the angiogenesis, VEGF expression, and growth tumor, but any effects on the normal tissues were observed (Gu et al., 2013). Meanwhile, the treatment with an anti-VEGF agent is reported to be effective in eliminating the abnormal vessels and retinal edema of diabetic retinopathy, but the inhibiting VEGF showed detrimental effects on the apoptosis of neuronal cells in STZ-diabetic rat retina (Park, Kim, & Park, 2014). In T1D-GT, NcVEGF+/mm2, NcCD31+/mm2 and MVD level in the PDL maintained similar to observed in NG rats associated to major preservation of periodontal tissues compared to T1D-W. We result suggested a major vascular response and healing during bacterial challenge in the diabetic periodontal tissue that received green tea. In addition, in previous study, the green tea intake reduces expression of the pro-inflammatory cytokine TNF-α and RANKL to normal levels in PDL of STZ-T1D rats reducing osteoclastic bone resorption (Gennaro et al., 2015) as also observed in periodontitis induced by ligature or Escherichia coli LPS in rats treated with EGCG polyphenol-green (Cho et al., 2013; Yoshinaga et al., 2014). Since, the TNF-α induces to VEGF, the reduction of TNF-α in diabetic rats by green tea can been responsible by VEGF to normal levels (Turer, Durmus, Balli, & Guven, 2017).

5 CONCLUSIONS

Long term of hyperglycaemia in T1D rats results in major dental plaque accumulation, vascular alterations, increase in VEGF expres-sion and advanced periodontal disease progression and tooth decay development. Green tea consumption is not able to totally inhibit the spontaneous development of periodontal disease and dental caries in T1D rats. However, green tea improves glycaemic control and main-tained the microvasculature and expression of VEGF to normal

levels, decreasing dental plaque accumulation and delaying the evolution of periodontal disease and caries. Thus, green tea can be used as a possible adjunct therapy to mechanical cleaning procedures principally in diabetic patients which have a risk of poor glycaemic control.

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TABLE AND FIGURES LEGENDS

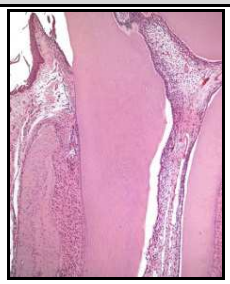
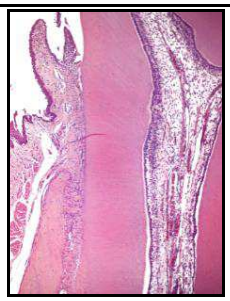
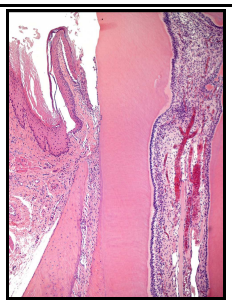
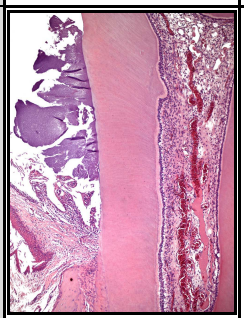
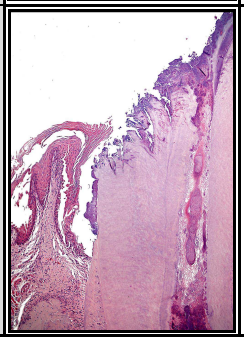
SCORES	PERIODONTAL DISEASE STAGES		TOOTH DECAY STAGES	
0		**HEALTHY** Healthy gum and periodontal ligament and bone anchor.		**HEALTHY** Healthy teeth, no visible plaque identified.
1		**GINGIVITIS** Focal inflammatory process limited to the gingival region without gingival recession.		**ENAMEL DECAY** Bit of white or brownish discoloration on the tooth's surface (first sign of tooth demineralization at the enamel level).
2		**EARLY PERIODONTITIS** Localized areas of gingival recession with formation of a periodontal pocket (at the cervical level) and loss of crestal bone height with grade 1 furcation involvement.		**DENTIN DECAY** Signs of resorption of dentin with/without reactionary dentin, but without pulp exposure or pulp involvement.
3		**MODERATE PERIODONTITIS** Gingival recession with loss of junctional epithelium and large periodontal pocket. Extensive loss of crestal bone height until 2/3 of the root with grade 2 furcation lesion (bone loss with substitution by connective tissue/inflammatory process).		**PULPITIS** Radicular/cornal dentin resorption with pulp involvement (advanced infection and intense inflammatory process).
4		**ADVANCED PERIODONTITIS** Extensive loss of crestal bone height up to 2/3 of root with grade 3 furcation involvement (gingival recession with bone exposure and accumulation of plaque with or without abscess).		**ABSCESS FORMATION** Radicular/coronal dentin resorption and/or periapical abscess.

Table 1: Pathological scores attributed for each stage of spontaneous periodontal disease and tooth decay in long-term of hyperglycaemia in T1D rats

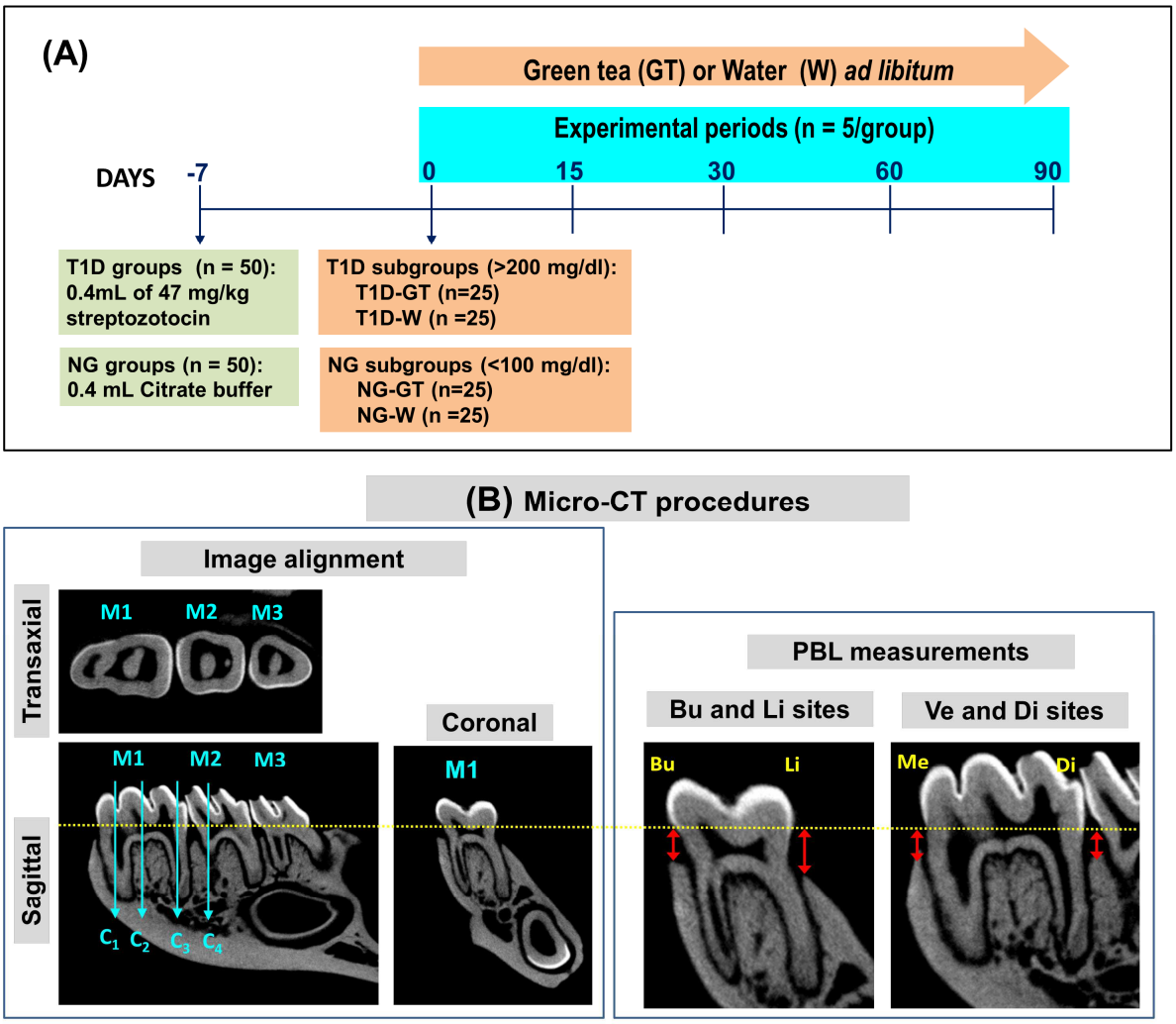
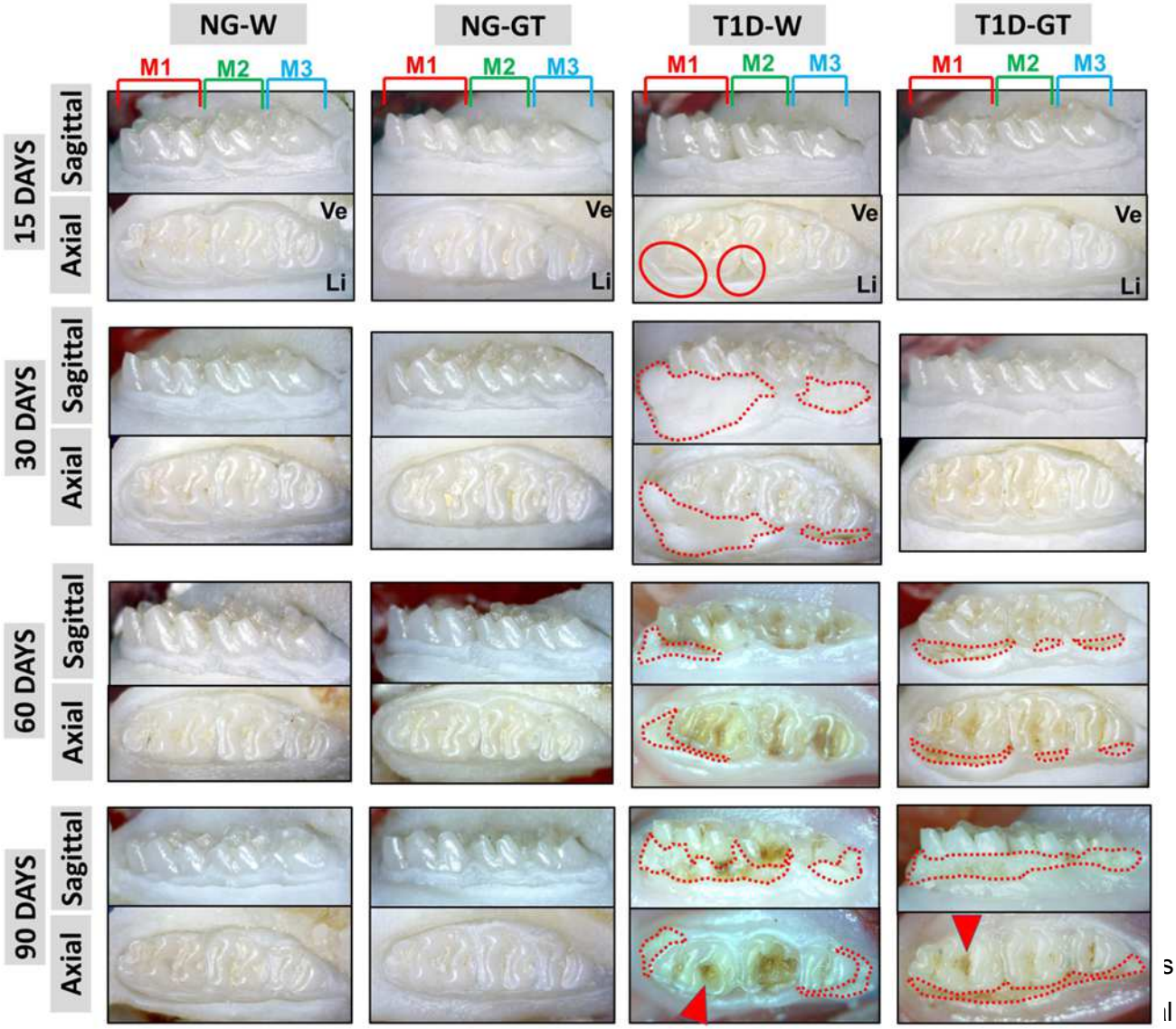
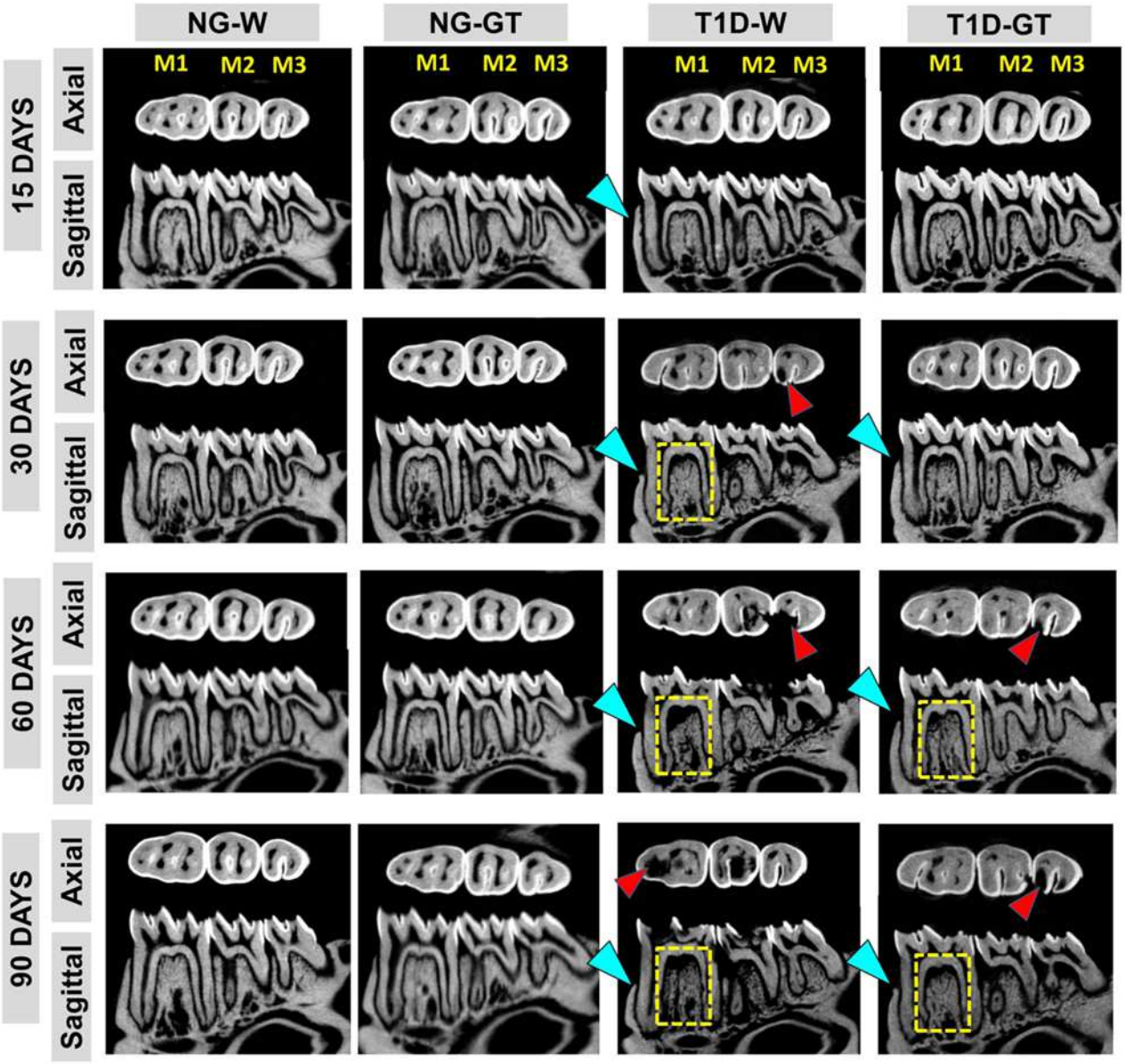


Figure 1. Study design and micro-CT procedures for determination of PBL in the CTAn program: Study design (A): Eighty rats were divided into two groups Normoglicemic (NG group) and diabetic (T1D group, induced by streptozotocin) 7 days before treatment. At day 0 two subgroups consumed green tea (NG-GT and T1D-GT subgroups) and two consumed water (NG-W and T1D-W subgroups) both ad libitum until the end of each experimental period. Micro-CT procedures (B): Image alignment: the molars of each hemimandible were realigned spatially in all planes so that CEJ of M1, M2 and M3 be at the same level (yellow dashed line). In the sagittal plane C1, C2, C3 and C4 represented the sections used for determination of buccal (Bu) and Lingual (Li) PBLs in the coronal plane and Mesial (Me) and Distal (Di) PBLs in the sagittal plane. PBL (red line) were measured by the distance between CEJ (yellow dashed line) and bone alveolar crest at each site.



plaque accumulation around lingual faces at 15 days (red circled area), major accumulation at 30 days (dot marked area in red), and less accumulation in the subsequent periods (60 and 90 days). This group also displays large caries lesions (red arrows) in the first and second molars. T1D-GT shows evident plaque accumulation in lingual faces after 60 days and small caries lesion at 90 days.



T1D-W shows initial resorption of alveolar bone crest in the mesial site at 15 days and continuous increase of PBL (blue arrowhead) between 30 and 90 days. After 30 days occurs a gradual reduction of trabecular bone (outlined yellow area) and formation of caries lesion (red arrow). T1D-GT exhibits slight periodontal bone loss only after 30 days and small areas of tooth decay at 60 days.

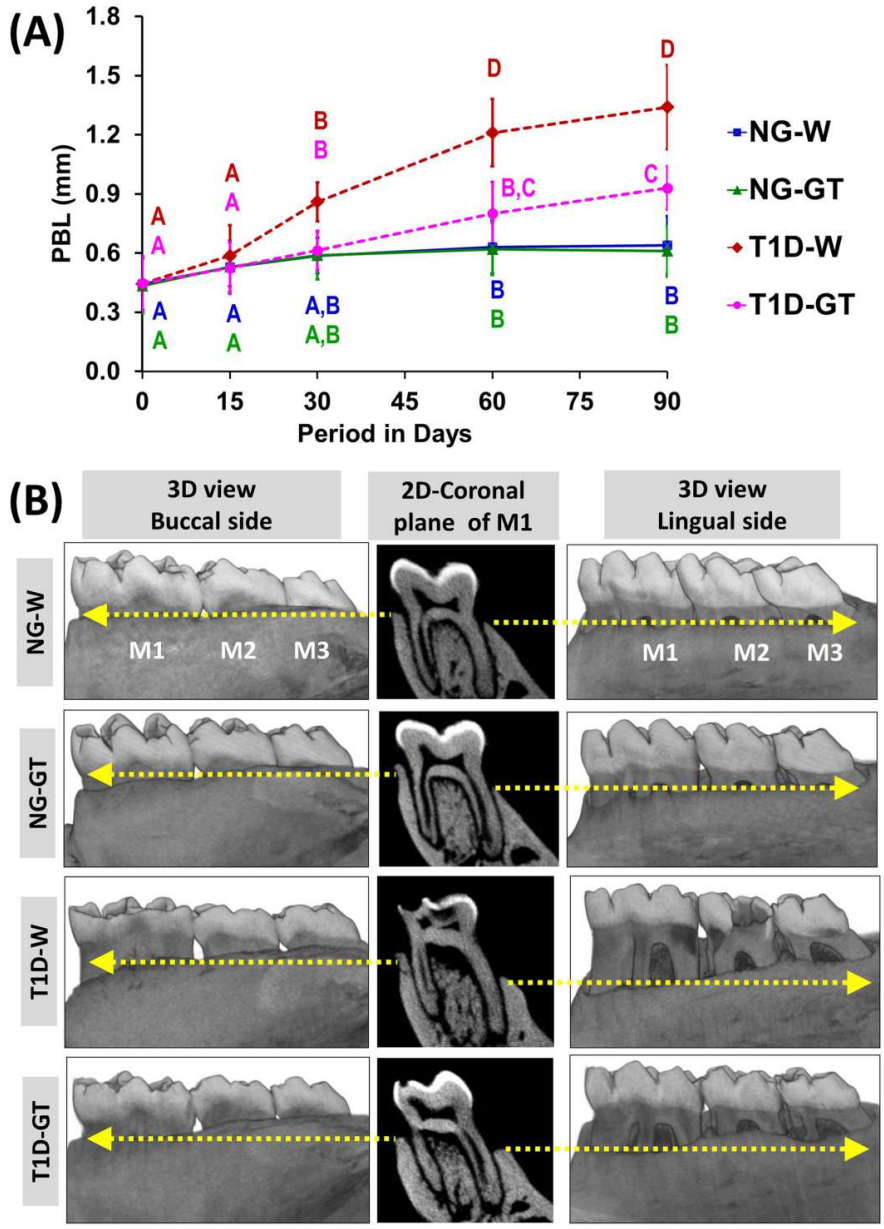


Figure 4. Graphic of periodontal bone level (PBL) of M1 (A) shows a gradual bone height loss with increase of PBL in the diabetic groups compared to normoglycemic groups. But, in T1D-GT the bone height loss/PBL were significantly smaller compared to T1D-W after 30 days. Different letters indicate significant differences ($P \leq 0.05$) among groups and periods. **Microtomographic images of the hemimandibles at 90 days (B)** show in 3D-imagens of buccal and lingual sides and 2D-coronal plane obtained of the M1 central region marked reduction of alveolar bone crest level (dashed yellow line) with extensive exposure of the root surfaces in diabetic groups (T1D-W and T1D-GT) in relation to normoglycemic (NG-W and NG-GT). 3D-images show that the reduction of bone crest level (dashed yellow line) was smaller in diabetic rats that received green tea (T1D-GT) compared to T1D-W, being more accentuated in lingual side.

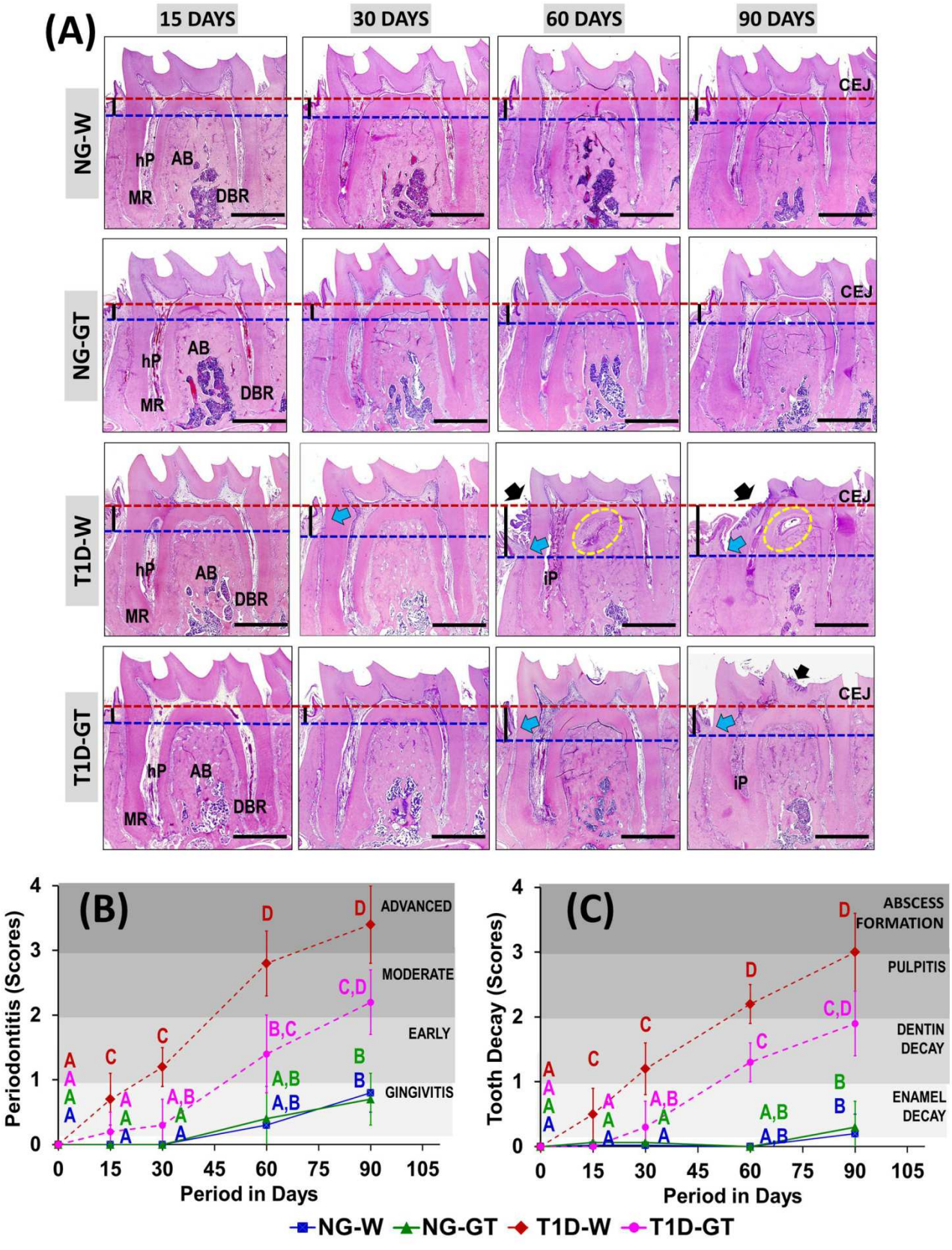

(A)
15 DAYS
30 DAYS
60 DAYS
90 DAYS
NG-W
NG-GT
T1D-W
T1D-GT
hP
AB
MR
DBR
CEJ
iP
(B)
Periodontitis (Scores)
ADVANCED
MODERATE
EARLY
GINGIVITIS
Period in Days
(C)
Tooth Decay (Scores)
ABSCESS FORMATION
PULPITIS
DENTIN DECAY
ENAMEL DECAY
Period in Days
NG-W
NG-GT
T1D-W
T1D-GT

Figure 5. Histopathological changes (A) and graphic evolution of scores attributed for periodontitis (B) and tooth decay (C) at different periods. NG-W and NG-GT show integrity of the periodontal and dental structures along the periods and small increase of PBL (black line), distance between alveolar bone (AB) and cement-enamel junction (CEJ), at 60 days. T1D-W at day 15 until day 30 shows gingival recession (blue arrow) and early periodontitis. After day 60 T1D-W molar shows dental calculus formations (black arrowhead) recovering exposed root with periodontal destruction, increase of PBL (black line), furcation lesion (dotted yellow line circle). Deep caries pulp with pulp inflammation (iP) and/or necrosis pulpar (nP) are more accentuated at day 90 showing picture of advanced periodontitis and tooth decay. In T1D-GT significant alveolar bone (AB) loss is evident only at 60 days, while in dentin and pulp structures are well preserved. At 90 days dentin caries (black arrow) and pulp inflammation (iP) are present. MR = mesial root; DBR = distal-buccal root and hP = healthy pulp; HE, x10 objective and Bar = 1000 μm. In the graphics different letters indicate significant differences ($P \leq 0.05$) among groups and periods.

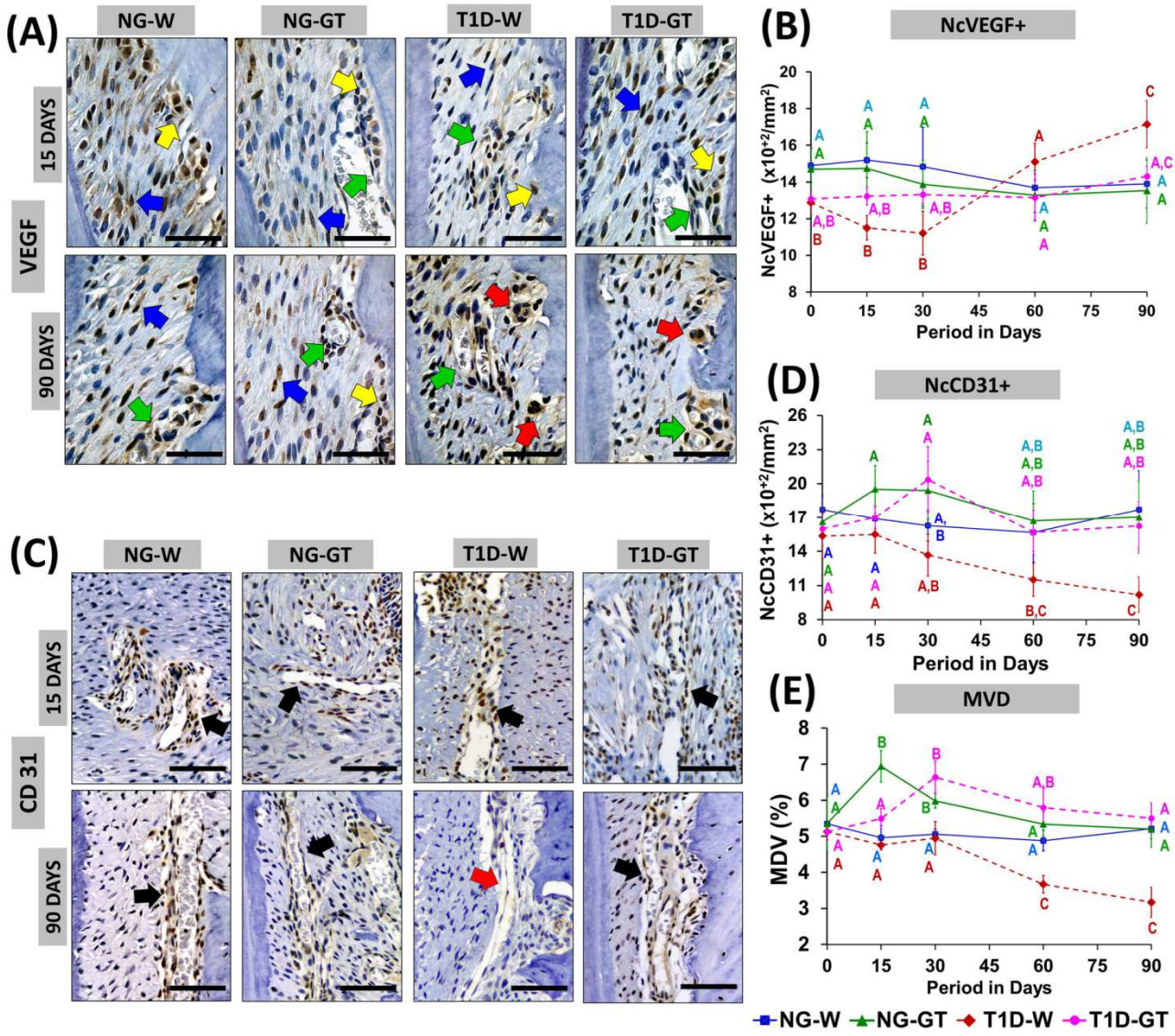


Figure 6. Immunolabeling patterns and quantitative evaluation for VEGF and CD31 and MDV in the periodontium. A) VEGF expression is present in Fibroblast (blue arrow), osteoblasts (yellow arrow), endothelial cells (green arrow) and osteoclasts (red arrow). Bar = 50 µm. B) NcVEGF+ graphic shows similar pattern in NG-W, NG-GT and T1D-GT, while in T1D-W, it decreases until 30 days and increases between 30 and 90 days. C) CD31 expression is evident in endothelial cells (black arrow). Bar = 120 µm. Note small diameter of vessel and weak CD31 immunolabeling in endothelial cells (red arrow) in T1D-W at 90 days. D) NcCD31+ graphic shows a reduction in T1D-W between 15 and 90 days. In the other groups no differences are observed during all experimental periods. E): MVD graphic shows constant values in T1D-W during all experimental periods, while NG-GT and T1D-GT show a peak at 15 and 30 days, respectively. In T1D-W a marked reduction of MDV is observed between 30 and 90 days. In the graphics different letters indicate significant differences ($P \leq 0.05$) among groups and periods.

2.2 ARTICLE 2 - Epigallocatechin gallate of green tea attenuates progression of periodontitis induced by ligature in diabetic rats.

Abstract

Aim: Verify if daily administration of EGCG attenuates alveolar bone loss in diabetic rats with periodontal disease induced by silk ligature. **Materials and Methods:** 120 *Wistar* rats were divided in: water treatment (NG-WT, n=20 and T1D-WT n=20), EGCG daily treatment (NG-EGCG, n=20 and T1D-EGCG, n=20) saline solution daily treatment (NG-Sham, n=20 and T1D-Sham, n=20). Periodontitis was induced by ligature around the right mandibular first molar 7 day after starting treatment. After 0, 7, 14 and 21 days (n=5 animals/period), the hemi-mandibles (n=5 subgroup) were collected. Scores of the degree of periodontal disease, PBL and BV/TV were analyzed. **Results:** Similar gradual increase in the total PBL was observed in all experimental groups until 14 days ($p>0.05$). At 21 days the total PBL of T1D-WT and T1D-Sham increase in mean 132% ($P<0.01$) while in the NG-WT, NG-Sham, NG-EGCG and T1D-EGCG remained similar. Between 14 and 21 days a significant ($p>0.01$) increase of BV/TV interradicular was observed in normoglicemic groups (69±5.5%) and T1D-EGCG (65±8.3%), while T1D-WT and T1D-Sham showed a tendency of reduction (49±8.3%), however no statistical differences was observed ($p>0.05$). The PD-scores in T1D-EGCG did not show statistical differences when compared to NG groups ($p=0.796$). **Conclusion:** Daily EGCG consumption has therapeutic effect on the periodontal disease in hyperglycemic condition, demonstrating antioxidant and anti-inflammatory action.

Keywords: Polyphenol Oxidase, Antioxidants, Diabetes Mellitus, Catechin,

Periodontal Diseases, Histological Techniques, X-Ray Microtomography

[*] Catanzaro DP, Mena Laura EE, Cestari TM, Mansano, BSDM, Garlet GP, Taga R, Assis GF. Green tea prevents vascular disturbs and attenuates periodontal breakdown in long-term hyperglycaemia in T1D rats. Journal of Clinical Periodontology (In preparation).

2.2 ARTICLE 1 – Epigallocatechin gallate of green tea attenuates progression of periodontitis induced by ligature in diabetic rats. (Appendix 2).

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CLINICAL RELEVANCE

Scientific rationale for the study: Diabetes is related to host response dysfunction that increases regulation of inflammatory mediators. Therefore, diabetes may induce periodontal disease during deregulation of the immune and inflammatory response.

Principal findings: Treatment with EGCG in diabetic rats provided early degree of periodontitis compared to untreated animals. Systemic administration of EGCG could have a therapeutic effect on damaged periodontal tissue through inhibition of cytokine expression responsible for the reduction in osteoblast formation, osteoclastic activity and collagen destruction.

Practical implications: EGCG can be used as an auxiliary therapeutic agent in periodontal disease in diabetic patients.

1 INTRODUCTION

Diabetes mellitus is a chronic disease inherited and / or acquired by a deficiency in insulin production by the pancreas or inefficacy of the insulin produced. Such deficiency results in increased blood glucose concentrations, which in turn cause damage to various body organs / systems (Organization, 2013). These include periodontal diseases that have been described as the sixth complication of diabetes mellitus (Loe, 1993). Currently, about 4% of adults receiving oral care are diabetic (Orso and Pagnoncelli, 2002; Sousa *et al.*, 2003; Negrato and Tarzia, 2010), stressing the importance of diabetes studies for periodontics. In the general context, diabetes is related to host response dysfunction that exacerbates the expression of intracellular signaling molecules, resulting in high regulation of inflammatory mediators. Therefore, diabetes may induce periodontal disease during deregulation

of the immune and inflammatory response against commensals of the periopathogenic microbiota. (Garlet *et al.*, 2013).

Periodontal disease, in the early period is asymptomatic, but with the aggravation of the disease the inflammation extends and results in tissue destruction and alveolar bone resorption leading to gingival erythema and edema, gingival bleeding, gingival recession, tooth mobility, periodontal pockets, suppuration and tooth loss (Preshaw *et al.*, 2012). The hyperglycemic condition accelerates periodontal destruction altering polymorphonuclear leukocyte function, collagen metabolism and vascular permeability, thus reducing viability and differentiation of cells in the periodontium, and altering microflora (Mealey, 1999; Lalla *et al.*, 2001; Hudson *et al.*, 2003).

Most of these tissue and cellular changes occur in the hyperglycemic state due to the formation of advanced glycation end products (AGEs - from english ingles, *Advanced Glycated End Products*). Through the generation of free radicals, formation of protein cross-links and interactions with cell receptors, AGEs promote, respectively, oxidative stress, morphofunctional changes and increased expression of inflammatory mediators. In addition, after inflammatory stimulation, as in periodontal disease, neutrophils, monocytes and macrophages produce myeloperoxidase and NADPH oxidase, which induce the formation of AGEs by amino acid oxidation. Locally generated AGEs interact with RAGEs (cell surface receptors) (Schmidt *et al.*, 1992), then initiate and propagate a RAGE-dependent inflammatory response.

Interestingly, in diabetes, the excessive expression of RAGE alters intracellular signaling, leading to alteration in gene expression and promoting the release of pro-inflammatory cytokines such as IL-1α, IL-6 e TNFα as well as free radicals that contribute to the complications of diabetes. It is noteworthy that NF-κβ is

activated in many inflammatory and neoplastic conditions by various cytokines. Consequently, NF-κβ Downregulates the expression of cytokines that mediate the autocrine cytokine release and amplification cycle of NF-κβ, controlling then the inflammatory reaction. These studies demonstrated AGE-RAGE leading to the exacerbated inflammatory response and periodontal tissue destruction in diabetes (Neumann *et al.*, 1999).

In a study conducted in our laboratory using an experimental model of spontaneous periodontitis in diabetic rats without use of ligature or inoculation of periopathogens, alveolar bone loss occurred concomitantly with the transient increase of osteoclasts (Claudino *et al.*, 2007). According to (Ogasawara *et al.*, 2004; Nagasawa *et al.*, 2007) this is due to the migration of inflammatory cells expressing RANKL into periodontal tissues. In addition, hyperglycaemia can modulate the RANKL / OPG ratio across the AGE / RAGE axis, tilting the balance for inflammation and tissue destruction. RANKL / OPG ratio in gingival fluid is high in poorly controlled diabetic patients with periodontitis compared to well controlled or non-diabetic individuals with similar periodontal condition (Santos *et al.*, 2010; Ribeiro *et al.*, 2011). These studies have proposed that hyperglycaemia may modulate the RANKL / OPG relationship in periodontal tissues. Additionally, the AGE-RAGE axis has also been suggested to contribute to osteoclastogenesis through increased RANKL expression and OPG down-regulation in various cell types (Ding *et al.*, 2006; Yoshida *et al.*, 2009).

Green tea is one of the most popular drinks in the world and has received considerable attention because of its beneficial effects on human health (Weisburger, 1999). Antioxidant anticancer, antimicrobial and anti-inflammatory properties of green tea have been well documented (Chen *et al.*, 2008). These effects are largely

attributed to the polyphenol contained in green tea, specifically, the (-) - epigallocatechin-3-gallate (EGCG). This molecule acts by interacting in various ways with biomolecules such as proteins, lipids and nucleic acids (Nozaki *et al.*, 2009). EGCG not only binds enzymes that act on DNA transcription activating molecules, but is also capable of binding directly to DNA and RNA (Balasubramanian and Eckert, 2004), protecting against free radical damage, ionization, ultraviolet radiation and DNA methylation that can induce the cancer cell (Suganuma *et al.*, 1996).

In addition, EGCG inhibits RANKL-induced osteoclast differentiation via suppression of NF-kβ transcriptional activity (Lee et al., 2009). On the other hand, it has been reported that AMPK (AMP-activated protein kinase) acts as a negative feedback regulator of RANKL-induced osteoclast formation (Lee *et al.*, 2010). Thus, AMPK may participate in both stimulation of bone formation and suppression of bone resorption. Several studies have also point EGCG suppress LPS-induced bone resorption by inhibiting IL-1β production, or directly by inhibiting osteoclastogenesis (Yun *et al.*, 2004; Rogers *et al.*, 2005; Yun *et al.*, 2007). Thus, our study aims to verify the anti-inflammatory and osteoblastogenic action of the EGECG in ligature induced periodontitis in diabetic rats.

2 MATERIAIS AND METHODS

2.1 Animals and experimental groups

The experimental protocol was performed in accordance with the Brazilian National Council for Animal Experimentation (CONCEA) and after the approval of the Animal Use Ethics Committee of Bauru Dental School - University of São Paulo

(CEUA/FOB-USP 0032/2013) (Annex 2). One hundred and twenty male Wistar rats (*Rattus norvergicus albinus*) with 60 days of age and a mean weight of 250g were housed in plastic cages and maintained at controlled conditions of temperature (22◦C±2◦C), humidity (55±10%) and light-dark cycles (12/12), with food and water *ad libitum*. Fourteen days before ligature (T-14), the animals were randomly assigned to two groups normoglicemic NG (n=60) and experimentally induced T1D (n=60) by intraperitoneal injection of 47 mg/kg of streptozotocin (Sigma Aldrich, St. Louis, MO, USA) diluted in citrate buffer. After confirmation of status diabetic (T-7), the rats were subdivided into six groups according to the treatment: a) T1D-EGCG (n = 20) and NG-EGCG (n = 20) received daily 100 mg/kg of EGCG (94% purity, Sunphenon® EGCG. Specialized Green Tea Extract Powder. Taiyo International, INC., Taiyo Kagaku C., Ltd. Minneapolis, MN - USA) (Annex 3) diluted just before administration in 1mL PBS by gavage; b) T1D-SHAM (n=20) and NG-SHAM (n=20) received only vehicle (PBS = 1mL) by gavage and c) T1D-WT and NG-WT (n=20) without treatment (Figure 1A). The experiments are reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Kilkenny *et al.*, 2012).

2.2 Ligature-induced periodontitis

After 7 days of the beginning of treatment (T0) the experimental periodontitis was performed under general anesthesia with intramuscular aplication of ketamine and xylazine (5 mg/kg and 0.1 mg/kg of body mass, respectively, both of Ceva®). A 3-0 Silk suture (Shalon®) was carefully placed around the cervix region of the right lower first molar. The ligatures were kept in subgingival positions during the

experimental periods of 0, 7, 14 and 21 days (n=5 per group and period) to temporal evaluation of periodontal disease by accumulation of dental plaque. Contralateral teeth were used as controls (healthy tissues) and presented similar results between groups. Figure 1A.

2.3 Hemimandibles collection

After each experimental period, the weight and fasting glucose levels (FGL) were measured. Subsequently, the rats were euthanized by overdose of anesthetic agent by intraperitoneal injection. The hemimandibles were removed and fixed in 10% phosphate buffered formalin solution at pH 7.2 for 1 week.

2.4 Micro-CT assessment and determination of periodontal bone level

The right and left hemimandibles were scanned in a microtomography scanner Skyscan 1176 (Bruker, Kontich, Belgium) at 50kV and 800μA with a resolution of 14μm per pixel and rotation of 180 degrees with steps of 0.8°. The images generated were reconstructed and reoriented spatially according to literature (Chang *et al.*, 2013), follows the sequence: 1) in the sagittal plane, the crowns from the first molar (M1) to the third molar (M3) were centrally and vertically positioned; 2) in the sagittal plane, the molar cemento-enamel junction (CEJ) were aligned horizontally at the same level; and 3) in the coronal plane, the CEJ in vestibular and lingual sites were aligned horizontally. The periodontal bone level (PBL) was determined by mean of the four distances from the CEJ to the alveolar bone crest (ABC) at the vestibular and lingual sites of M1 and M2 in the coronal plane, while mesial and distal PBL was measured by the distance between CEJ and bone alveolar crest at each site (see

Fig. 1B1). M1 interradicular bone density (BV / TV) was obtained in sagittal plane-aligned images using CTAn software (SkyScan, Belgium). Brief, in the 245 imagens/slices (490μm of depth) containing interadicular region between mesial and distal roots the region interest (ROI) was manually selected as illustrated in the Figure 1B2. The interradicular bone density of each M1 was determined by the percentage (%) of tissue evaluated.

2.5 Histological processing and histopathological analysis

The hemimandibles were decalcified with 4.13% ethylenediamine tetra acetic acid (EDTA) 7.2 pH for 8 weeks and processed histologically for embedding in Histosec (HistosecTM, Merck KGaA - Darmstadt, Germany). Longitudinal 4μm-thick serial sections were obtained and placed on silane-coated glass slides (Dako-Japan Co.,Ltd, Kyoto, Japan).

For histopathogical evolution of periodontal disease after ligature, three sections were stained with hematoxylin and eosin per hemimandible. One section showed the central portion of the coronal and radicular pulp chamber of the first molar (Figure 2A, S_2), and other two serial-sections at 150 μm of central section, one at lingual position (Figure 2A, S_1) and other at vestibular position (Figure 2A, S_3) were evaluate. The analyzes were performed by a dental surgeon, slides were further scanned into high-resolution images using the Aperio Scanscope CS Slide Scanner (Aperio Technologies Inc, Vista, CA, USA). All digital images obtained in.svs format were visualized with ImageScope software (Aperio Technologies Inc). For each M1, the periodontal index ranges from 0 to 4 was determinated scores: 0 no periodontal disease (Figure 2C); 1 gengivitis (Fig. 2D); 2 early periodontitis (Fig. 2E); 3 moderate

periodontitis (Fig. 2F) and 4 severe periodontitis (Fig. G) as describe and illustrated in the Fig. 2 (A-G).

2.5 Tartrate-Resistant Acid Phosphatase (TRAP) Immunostain

Three semi-serial sections per mandible were submitted to immunohischemistry procedure for detection of TRAP cells using a goat polyclonal anti-human peptide N-17(sc-30832, Santa Cruz Biotechnology, Inc.®) in according to previous study (Mena Laura *et al.*, 2019). The immunostaining pattern of TRAP cells was evaluated in Aperio Scanscope CS Slide Scanner (Aperio Technologies Inc, Vista, CA, USA). All digital images obtained in .svs format were visualized with ImageScope software (Aperio Technologies Inc). The area total of alveolar bone in the distal, mesial and interradicular regions was determined and the number total of TRAP+ cells were quantified manually. The TRAP+ cells number was calculated per mm^2 of alveolar bone

2.6 STATISTICAL ANALYSIS

All statistical analyses were made with Prism 5.00 software for Windows (GraphPad Software, Inc., California, USA). First, all data of PBL, BV/TV, PD-scores and TRAP+ cell numbers were submitted to normality test (Kolmorov-Smirnov). Since this parameter was satisfied, the data were compared among periods and experimental groups by one-way analysis of variance (ANOVA), and the means contrasted by Tukey´s test. For all cases, $p<0.05$ was established as the level of significance.

3. RESULTS

3.1. Clinical Assessments for Body mass and Fasting blood glucose

Statistical data show a similar initial body mass (T-14) among groups, being on average 282.97 ± 99.84g. Between 0 and 14 days after ligature (T0 -T14), body mass of NG-WT, NG-Sham and NG-EGCG animals showed no differences among groups and periods ($p>0.05$) being on average 314.67 ± 3.6 g at 14 days. In the same period, body mass of diabetic animals, T1D-WT, T1D-Sham e T1D-EGCG, similarly reduced 28.36% showing no difference among groups (mean 202.67± 7.85g, $p>0.05$) however 35.6% lower than normoglycemic ($P<0.01$). The difference between body mass in NG versus T1D animals remained until 21 days.

Fasting blood glucose of animals at T-7 averaged 76.13 ± 10.44 mg / dL in normoglycemic rats and 454.4 ± 112.87 mg / dL in diabetics; glycaemia remained constant until the final 21 days.

3.2. Determination of periodontal bone level (PBL) and BV/TV interradicular in M1 roots by micro-CT analysis

The periodontal morphology, periodontal bone level (PBL) and volume density interradicular (BV/TV) of the M1 of right mandible at 0 days (before of ligature) were similar to the M1 of left mandibule (contralateral side without ligature) among groups and periods ($p\geq0.7$). Thus, the Figure 3 showed only representative 3D micro-CT images of right mandible at 0 and 7, 14 and 21 days after M1 ligadure (Figs. 3A–X), as well as, the respective mean ± DP graphs obtained for the PBL (Fig. 3Y), and BV/TV interradicular (Fig. 3Z).

At T0, in all experimental groups (A, E, I, M, Q and U) a similar total PBL (mean of 463 ± 28μm, $p>0.05$). PBL was higher in the lingual side (mean of 764±53.7μm) than in vestibular side (mean of 376±14.4μm) and higher in mesial side (mean of 419±15.1μm) than distal (mean of 294±29.1μm). After ligature, a similar gradual increase of 235 ± 66% ($p<0.001$) in the total PBL was observed in all experimental groups until 14 days ($p>0.05$). This increase was of 295 ± 25 μm (139%) in lingual side, 583 ± 77μm (255%) in vestibular side, 810 ± 51μm 293% in mesial side and 445 ± 40μm (251%) in distal side. At 21 days the total PBL values maintained similar to 14 days ($p>0.05$) in the NG-WT (972±167μm), NG-Sham (1120±118μm), NG-EGCG (968±153μm) and T1D-EGCG (1087±122μm), while in the T1D-WT and T1D-Sham the total PBL increase in mean 132% (1371±134μm and 1384±129μm, respectively, $p<0.01$). See Graphic Y from Figure 3.

Similarity, BV/TV interrradicular was similar among groups at 0 days ($p>0.05$), occupying in mean 81.45 ± 6% of the space. Seven days after ligature a similar interradicular BV/TV reduction was observed in all experimental groups (mean of 56 ± 8.2%, $p>0.05$), and it remained constant until 14 days. Between 14 and 21 days a significant ($p>0.01$) increase of BV/TV interradicular was observed in the NG-WT (72±4.6%), NG-Sham (68±5.8%), NG-EGCG (67±6.0%) and T1D-EGCG (65±8.3%, while T1D-WT and T1D-Sham showed a tendency of reduction, however no statistical differences was observed (50±5.5% and 48±7.4%, $p>0.05$). See Graphic Z from Figure 3.

3.3. Level of Periodontal Disease (PD-score) in M1 root

The histological images (Figs. 4A–L and 5A-L) and graphics of Pearson correlation of PD-score attributed for 3 histological sections for each sample (Figs.

4M-O and 5M-O) showed a greater loss of periodontal structures after M1 ligadure in T1D-WT and T1D-Sham than T1D-EGCG.

At T0 (without ligature), in NG groups (Figs.4A, E and I) and T1D groups (Figs. 5A, E and I), all evaluated M1 showed absence of histological evidence of periodontal inflammation and no evident anatomical change in periodontium. Thus, in this period all M1 received PD-score 0. After ligature alterations in the periodontal structures, such as inflammatory process, pocket's epithelium, destruction of the collagenous periodontal ligament and bone resorption were observed in all groups experimental. However, in T1D rats (Figs 5B-D, 5F-H and 5J-L) these events were more exacerbated than in normoglycemic rats (Figs. 4B-D, 4F-H and 4J-L). With respect to normoglycemics rats, a similar histological picture of periodontal disease was observed among the groups, NG-WT (Figs. 4B-D), NG-Sham (Figs. 4F-H) and NG-EGCG (Figs. 4J-L) ($p=0.845$). In all cases (100%), inflammatory process was moderate and restricted to marginal gingiva leading to small gingival recession and destruction of periodontal fibers and alveolar bone crest in less than a 1/3 of the root. In the furcation area, the PDL and bone tissues destruction were replaced by connective tissue and non-exposed furcation area occurred at 7 days (compare Figs. 4B, 4E and 4J). In this period, the average PD-score was of 2.3 or early periodontitis (see Figs. 4B, F and J and scores in the Graphs of Figs. 4M-O). Between 14 and 21 days, only 7 cases received a PD-score equal to or greater than 3, two in NG-WT (20%) (Graph of Fig. 4M), three in NG-Sham (30%) (Graph of Fig. 4N) and two in NG-EGCG (20%) (Graph of Fig. 4O), thus, in no case PD-score 4 was observed.

Although PD-scores did not showed statistical differences among NG and T1D groups at 7 ($p>0.7169$) and 14 days ($p=0.2568$),10 cases (50%), four in T1D-WT (40%, graph of Fig. 5M) and six in T1D-Sham (60%, graph of Fig. 5N) showed PD-

score ≥3, being 3 cases (30%) at 7 days of ligature. However, in any case T1D-EGCG (graph of Fig. 5O) exhibited PD-score ≥3. This higher aggressiveness of periodontal disease in T1D compared to NG and protective effect of EGCG in T1D-EGCG were statistically confirmed after 21 days of ligature (p=0.0003). In this period, all cases in T1D-WT (Graph of Fig. 5M) and T1D-Sham (Graph of Fig. 5N) received score ≥3 (100% of cases). Among them, four cases (40%) received the maximum score (4) showing a long periodontal pocket, extensive gum recession, large vertical/horizontal bone loss and dental motility, as well as dentin and root caries (see Figs. 5D and 5H). In relation to T1D-EGCG only one case received PD-score of 3.33 (20%), while the other 4 cases (60%), the PD-score was small than 2.64 (see histological aspect in the Fig. 5L). The PD-scores in T1D-EGCG did not show statistical differences when compared to NG groups (p=0.796).

3.4. Level of TRAP+ cells in the alveolar bone of M1

The localization and quantitative expression of immunolabeled TRAP cells were evaluated in histological sections obtained in sagittal plane of M1. The ligature was knotted on the mesial side of the tooth, causing an exacerbated loss of periodontal structures as well as cervical root resorption. Thus, the periodontal changes during ligature was morphological characterized adjacent to proximal region (Fig. 6) and interradicular region (Fig. 7). However, the number of TRAP+ cells/mm^2 in alveolar bone was determinate between the mesial and distal roots. At 0 days, the integrity of periodontal and dental structures was observed in all experimental groups and mean of TRAP+cells number was of 1.8±1.3/mm^2 of alveolar bone (p=0.955).

At 7 days, in all groups, gingival and subepitelial connective tissues inflammation were evident (Fig. 6). Additionally, an apical migration of the alveolar

bone crest accompanied by epithelial attachment loss of the gingiva also occurred (see detais of Figs. B, C and D). On the furcation region, an initial level of furcation lesion with loss of the characteristic organized periodontal fibers arrangements and substituted by disorganized inflammatory connective tissue and presence of TRAP+ resorptive cells was observed (see details in the Figs.A-F). In this period, a significant increase of TRAP+cells number occurred in NG-WT (925%, 18.5±7.8 cell/mm^2, $p<0.05$), NG-Sham (804%, 15.3±8.1 cell/mm^2, $p<0.01$), T1D-WT (1231%, 30.6±11.2 cell/mm^2, $p<0.001$) and T1D-Sham (1734%, 30.8±7.3 cell/mm^2, $p<0.001$) and no statistically significant difference between them was detected ($p<0.05$). However, in NG-EGCG group between 0 and 7 days, the mean values of TRAP+ cells numbers showed an increase of 315%, but no statistical differences was detected.

At 14 days, TRAP+ cells numbers showed a similar reduction in the NG-WT (63%, 6.9±5.5 cell/mm^2), NG-Sham (52%, 7.2±2.7 cell/mm^2), T1D-WT (67%, 10.1±2 cell/mm^2) and T1D-Sham (60%, 12.2±14.2 cell/mm^2); while in NG-EGCG a peak of labeled cells (106%, 13.4±8 cell/mm^2) was observed. At 14 and 21 days, in all experimental groups occurred alveolar crest apical migration, epithelial attachment loss, and the TRAP+ cell numbers were similar among groups ($p=0.4619$ and $p=0.04388$, respectively). However, in all NG groups and T1D-EGCG the inflammatory process was diffuse and a few osteoclastic activity, restricted to cervical region, was associated to a great bone formation areas and reorganization of collagen fibers (see details in the Fig. H-J and M; N-P and S). Nonetheless, T1D-WT and T1D-Sham progressive periodontal breackdown occurred displaying a high gingival recession and radicular resorption until the apical region with presence of necrotic tissues above the epithelium. Although, advanced of furcation lesions grade 2 and 3 associated to intense inflammatory process, necrotic tissues above epithelial

tissue were present in all sample, a high bone formation and rarely TRAP+ cells also were observed.

4 DISCUSSION

In previous work (GENNARO et al., 2015), we found that in diabetic rats, ad libitum consumption of green tea slowed the evolution of diabetes and periodontal disease spontaneously developed in the animals. These were attributed to the polyphenols contained in green tea, which led to the reduction of proinflammatory and osteoclastogenic cytokines. Considering to the difficulty of controlling the amount and quality of the drug to be taken in tea form, we propose to ingest controlled amounts of the most active ingredient of green tea, EGCG, during the therapy of periodontal disease. In the current work, we observed that daily consumption of 25mg / kg EGCG in normoglycemic animals did not change the degree of ligature-induced periodontal disease whereas in diabetic rats EGCG promoted greater preservation of periodontal structures. Systemic administration of EGCG could have a therapeutic effect on damaged periodontal tissue. It inhibited cytokine expression, including TNF, IL-1β and IL-6 as a responde to the reduction of osteoclast formation, osteoclastic activity and collagen destruction (Okada and Murakami, 1998; Cho et al., 2013). IL-1β has been associated with inflammatory cell migration and osteoclastogenesis. Macrophages, which secrete large amounts of IL-1β, are found in high numbers in sites with periodontal diseases (Lagha and Grenier, 2019). The microtomographic and the periodontal level score results showed that the dental and periodontal tissues remained healthy in the T1D-EGCG groups along the experimentation. This same pattern in response to treatment was observed in the

micro-ct evaluation at 21 days the total PBL values being similar to that of at14 days (p> 0.05) in the NG-WT, NG-Sham, NG-EGCG and T1D-EGCG, while in the T1D-WT and T1D-Sham the total PBL increase in mean 132% (P <0.01). Similarity, BV/TV interrradicular at 14 and 21 days had a significant (p>0.01) increase of BV/TV interradicular in the NG-WT, NG-Sham, NG-EGCG and T1D-EGCG, while T1D-WT and T1D-Sham showed a tendency of density reduction, however no statistical differences was observed (p>0.05).

Gingivitis progression into advanced periodontitis with extensive vertical bone loss exposing the root apex was observed in T1D-WT and T1D-Sham at 14 and 21 days. Concomitantly, evolution of furcation injury also observed. Treatment with EGCG in T1D-EGCG limited it to early degree of periodontitis when compared to T1D-WT and T1D-Sham. We observed that the first signs of periodontal disease were already apparent within 7 days of ligature, however periodontal disease and disease progressions were less intense when compared to T1D-W and T1D-Sham. These results show an inverse relation between EGCG intake and periodontal disease progression, which has not been cited in the literature under hyperglycemic conditions, or with green tea use (Gadagi et al., 2013; Gennaro et al., 2015; Catanzaro et al., 2018).

In the initial 7-day period, the T1D-WT and T1D-Sham animals already, presented formation gingival recession with gradual increase of periodontal lesions until 21 days, with extensive gingival recession, with loss of junctional epithelium, periodontal pockets bone loss to, -2/3 of the root and dentin root resorption areas. However, T1D-EGCG showed only early degree of periodontal disease, even at 21 days (Lagha and Grenier, 2019), explain TNF-α may exert deleterious effects through the amplification of the inflammatory process and the disruption of the keratinocyte

barrier. Also show that tea polyphenols attenuate the gingival epithelial barrier dysfunction caused by TNF-α and modulate the inflammatory host response. Given the fact that pathological inflammation is, associated with periodontitis, it involves a loss of tolerance and / or regulatory processes, the ability of tea polyphenols to attenuate inflammatory processes suggests that they may be promising as a preventive or therapeutic agent for to be used as anti-cytokine therapies.

5 CONCLUSIONS

After treatment with EGCG, diabetic and normoglycemic rats with ligature-induced periodontal disease did not change weight and glycemia over the periods, indicating that this polyphenol does not interfere with the diabetic condition of the animals.

Regarding the periodontal morphology analyzed by mico-ct, PD-score in histological analysis and TRAP + cells by immunohistochemistry, EGCG-treated diabetic animals show better recovery from periodontal disease than normoglycemic animals also treated.

Thus, systemic administration of EGCG could have a therapeutic effect on damaged periodontal tissue through inhibition of inflammatory cytokines that are responsible for the reduction in osteoblast formation, osteoclastic activity and collagen destruction. EGCG can be an auxiliary therapeutic agent for periodontal disease in diabetic patients.

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TABLE AND FIGURES LEGENDS

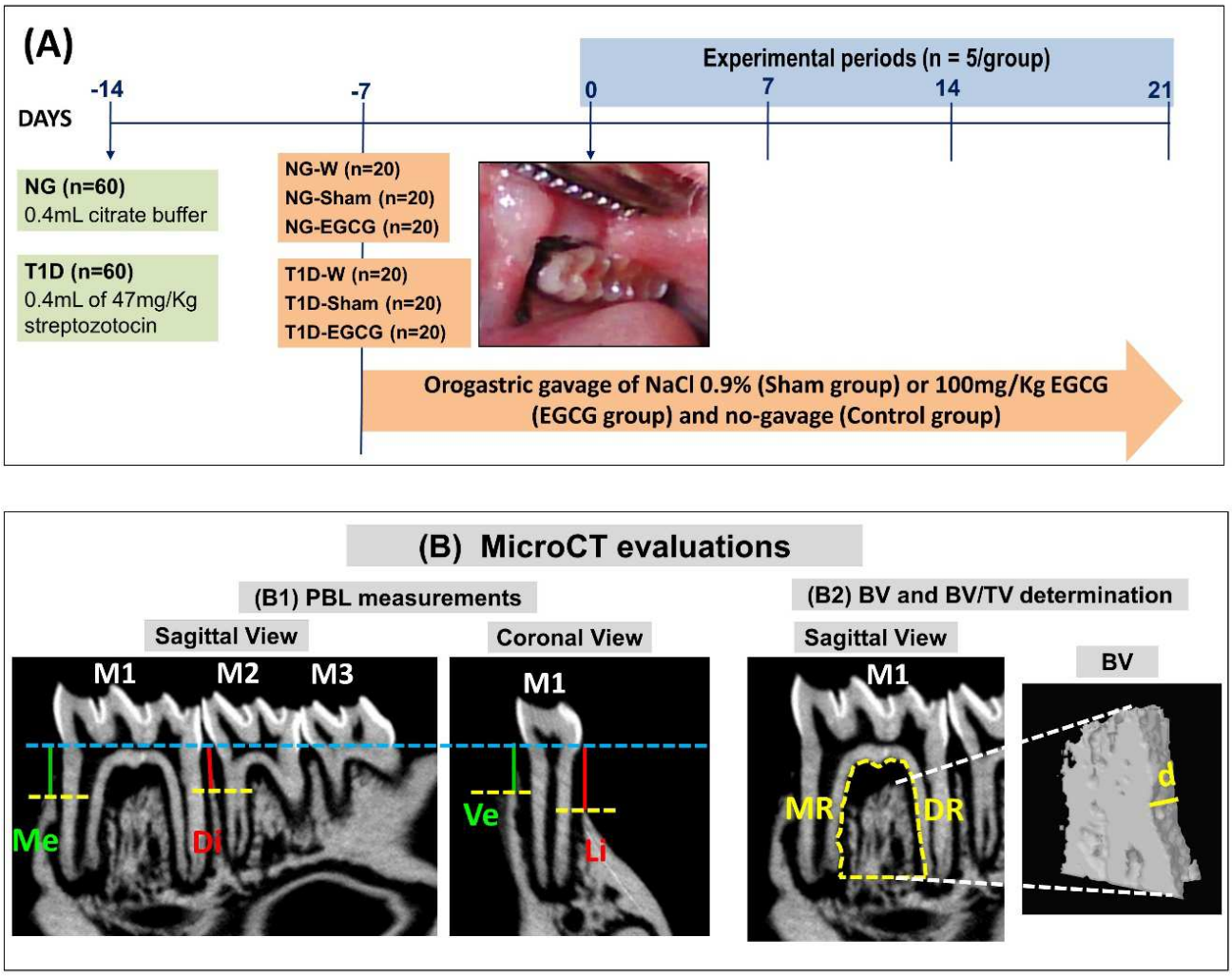

Figure 1: Study design (A): One hundred and twenty rats were divided into two groups Normoglicemic (NG group, n=60) and diabetic (T1D group, n=60 induced by streptozotocin) 14 days before treatment (-14). Seven days later (-7), after diabetic confirmation, two subgroups received EGCG (NG-EGCG and T1D-EGCG subgroups), two, saline solution (NG-Sham and T1D-Sham) and two no treatment (NG-WT and T1D-WT subgroups). All the groups received water *ad libitum*. After 7days, at day 0, a Silk ligature was placed around the mandibular right first molar cervix. **Micro-CT bone level (PBL) determination in CTAn program (B1):** Mandibles were aligned in all spatial planes so that CEJ of M1, M2 and M3 are at the same level (blue dashed line). In sagittal sections, around M_1, Mesial (Me) and Distal (Di) PBL measurements as distances between CEJ (blue dashed line) and bone alveolar crest (yellow lines); Similarly, in coronal sections, Vestibular (Ve) and Lingual (Li) PBL. Interradicular bone density (BV / TV) (B2): was determined in slices from the interadicular region between MR and DR with a depth (d) of 490µm.

(Fig.) Score	Level of Periodontal disease
(C) Normal Score 0	- Alveolar bone crest (BC, yellow traced line), cementum-enamel junction (CEJ, blue traced line), gingiva (green arrow) and alveolar bone are clinically healthy; - Small height between CEJ and BC (black arrow).
(D) Gengivitis Score 1	- Gingivitis or gingival recession, presence of junctional epithelium and no attachment loss (blue arrow); - Absence of gingival pocket or pseudopocket (gingival hyperplasia).
(E) Early Periodontitis Socre 2	- Gingival recession (blue arrow); - Horizontal bone loss (white arrow), alveolar bone crest or interdental septum loss until 1/3 of root (yellow traced line) ; without periodontal pocket formation; - Furcation grade 1 filled by connective tissue without foci of inflammatory (white arrow).
(F) Moderate Periodontitis Score 3	- Gingival recession (blue arrow) with or without loss of junctional epithelium; - Periodontal pocket (red traced line) and extensive horizontal (black arrow) and vertical bone loss up to 2/3 of root (grey arrow), with furcation grade 2 or 3 filled by connective tissue with inflammatory process; - Few root resorption areas.
(G) Advanced Periodontitis Score 4	- Extensive gingival recession (blue arrow) with loss of junctional epithelium; - Deep periodontal pocket with advanced bone loss beyond 2/3 of root; - Grade 3 or 4 furcation (grey arrow); - Areas of dentinal root resorption.

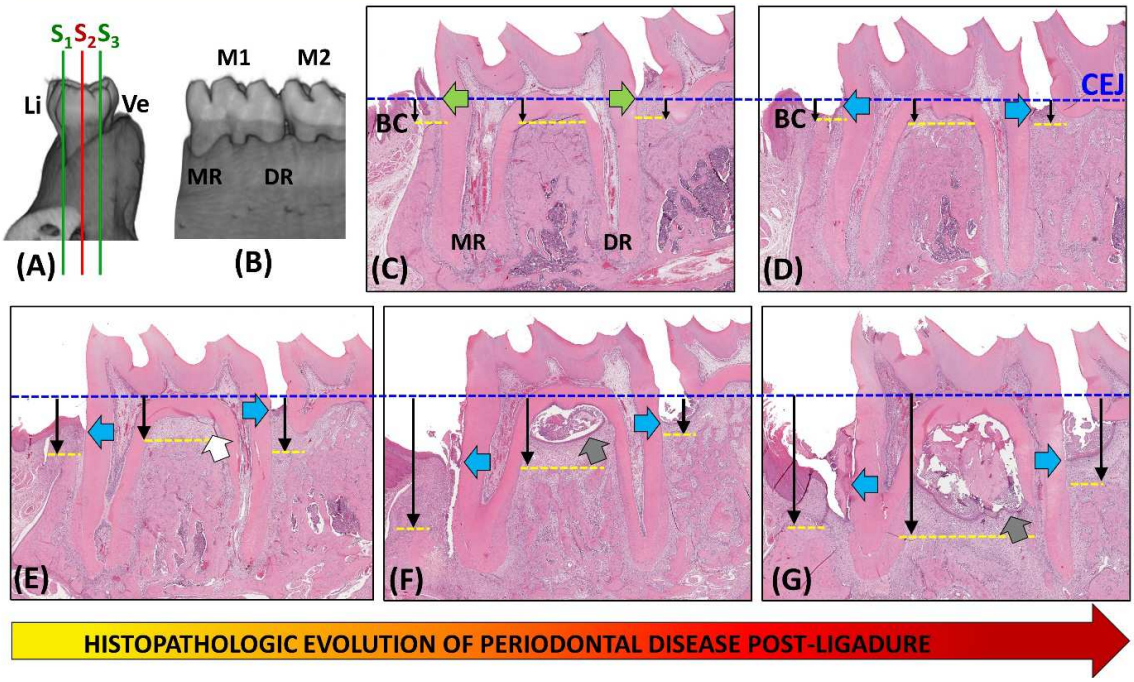

Figure 2: Post-ligature periodontitis evolution showing morphological aspects in longitudinal sections of the first molar according to periodontal scores: Three sections stained with hematoxylin and eosin per hemimandible, one section showing the central portion of the coronal and radicular pulp chamber of the first molar (M1) (S2) and two vestibular and lingual serial-sections at 150 µm from S2, (A, S1 and S3). Mesial Root (MR) and Distal Root (DR) of M1 are represented in B. C) Score 0 as healthy tissues and small height between the CEJ (blue dashed line) and BC (yellow dashed lines); D) Score 1 (Gingivitis), showing limited inflammatory process in the gingival region (blue arrow); E) Score 2 (Early Periodontitis), gingival recession and horizontal bone loss (black arrow) with presence of grade 1 furcation lesion (white arrow); F) Score 3 (Moderate Periodontitis), showing gingival recession with presence of periodontal pocket up to 2/3 root and horizontal mass loss in alveolar bone crest and interdental septum to 2/3 root with grade 2 lesion (grey arrow) ; G) Score 4 (Advanced Periodontitis), showing extensive gingival recession with loss of junctional epithelium and presence of deep periodontal pocket above 2/3 of the root, in addition to advanced vertical bone loss from the alveolar bone crest and interdental septum to a lesion root media region grade 3 and areas of root dentin resorption (grey arrow).

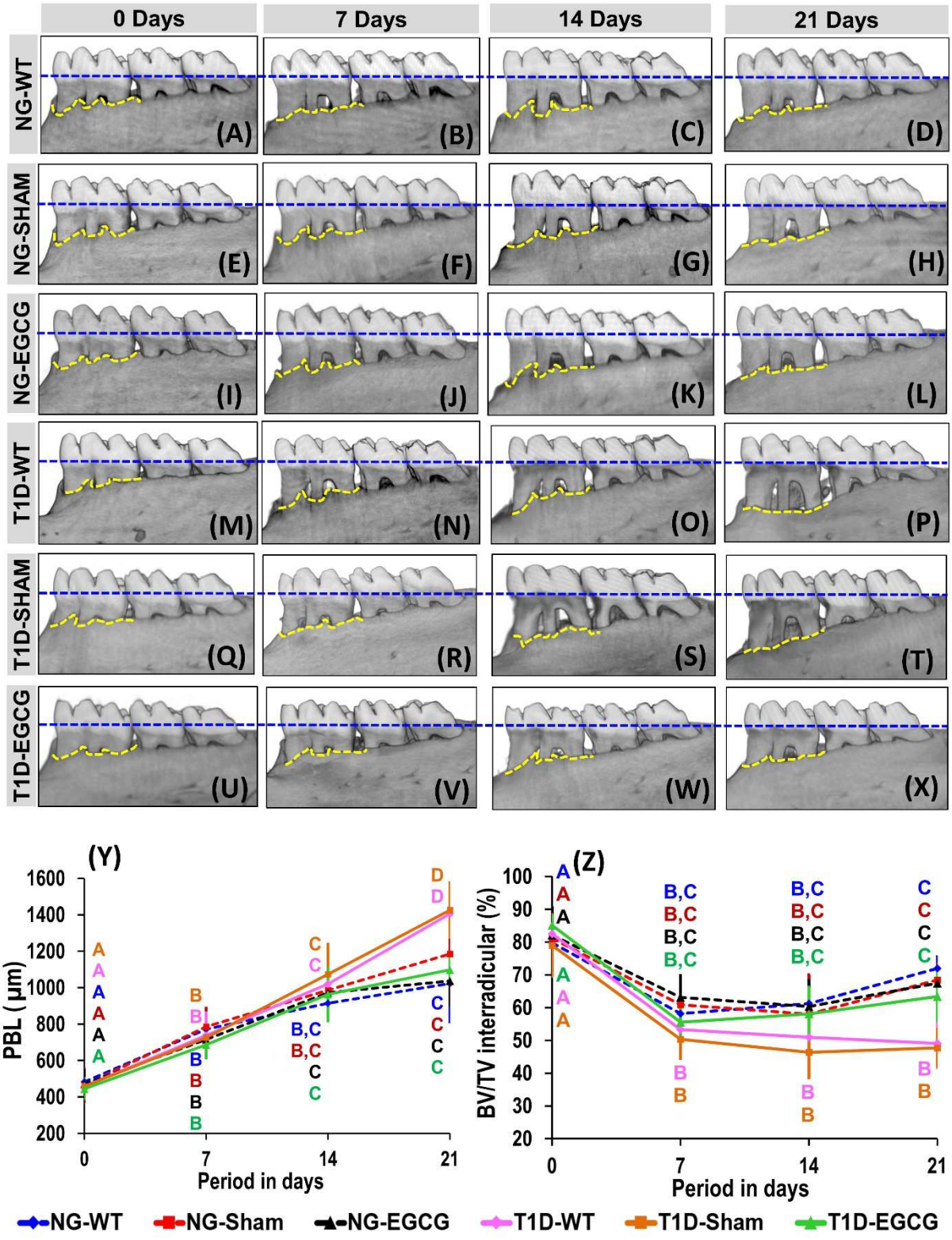

Figure 3: X-ray microtomographic images (A - X). Average periodontal bone level (PBL) obtained from four distinct sides: vestibular, lingual, mesial and distal (Y). Interradicular BV/TV mean comparisons among groups (Z). Distances were measured between the CEJ (blue dashed line) and BC (yellow dashed lines) at the vestibular and lingual sites of M1 in the sagital plane. Two way ANOVA and Tukey test (P≥0,005).

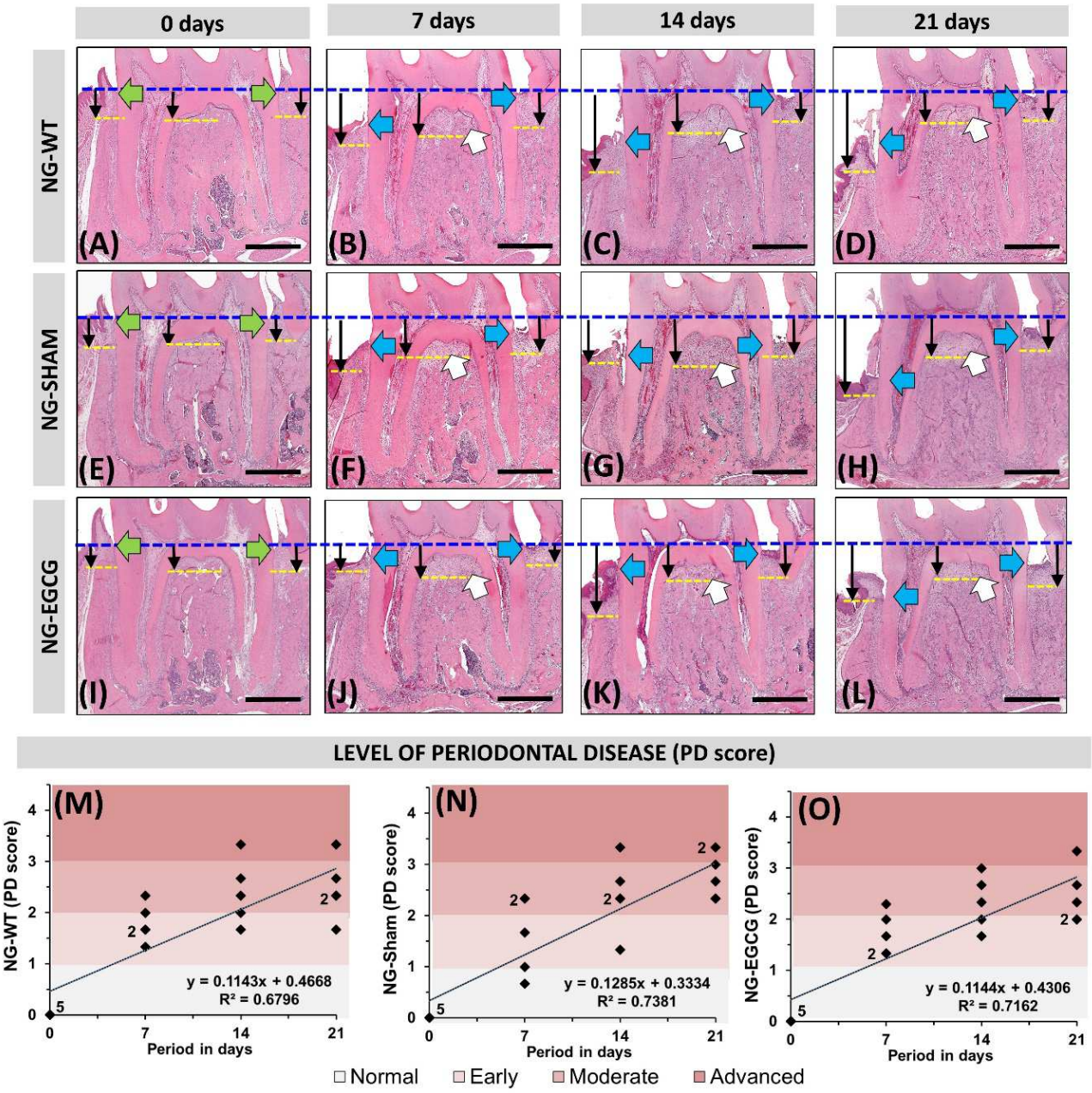

Figure 4. HE representative images (A-L) and graphic of Pearson correlation of Level of Periodontal Disease (PD-score) in M1 root of normoglycemic groups. HE images (A-L) and graphics of Pearson correlation of PD-score in NG-WT (M), NG-Sham (N) and NG-EGCG (O) show a progressive periodontal breakdown during time until the end of the periods. Gingiva clinically healthy (green arrow). Gingivitis or gingival recession (blue arrow). Horizontal bone loss, alveolar bone crest or interdental septum loss until 1/3 of root (white arrow). ST Barr = 1mm.

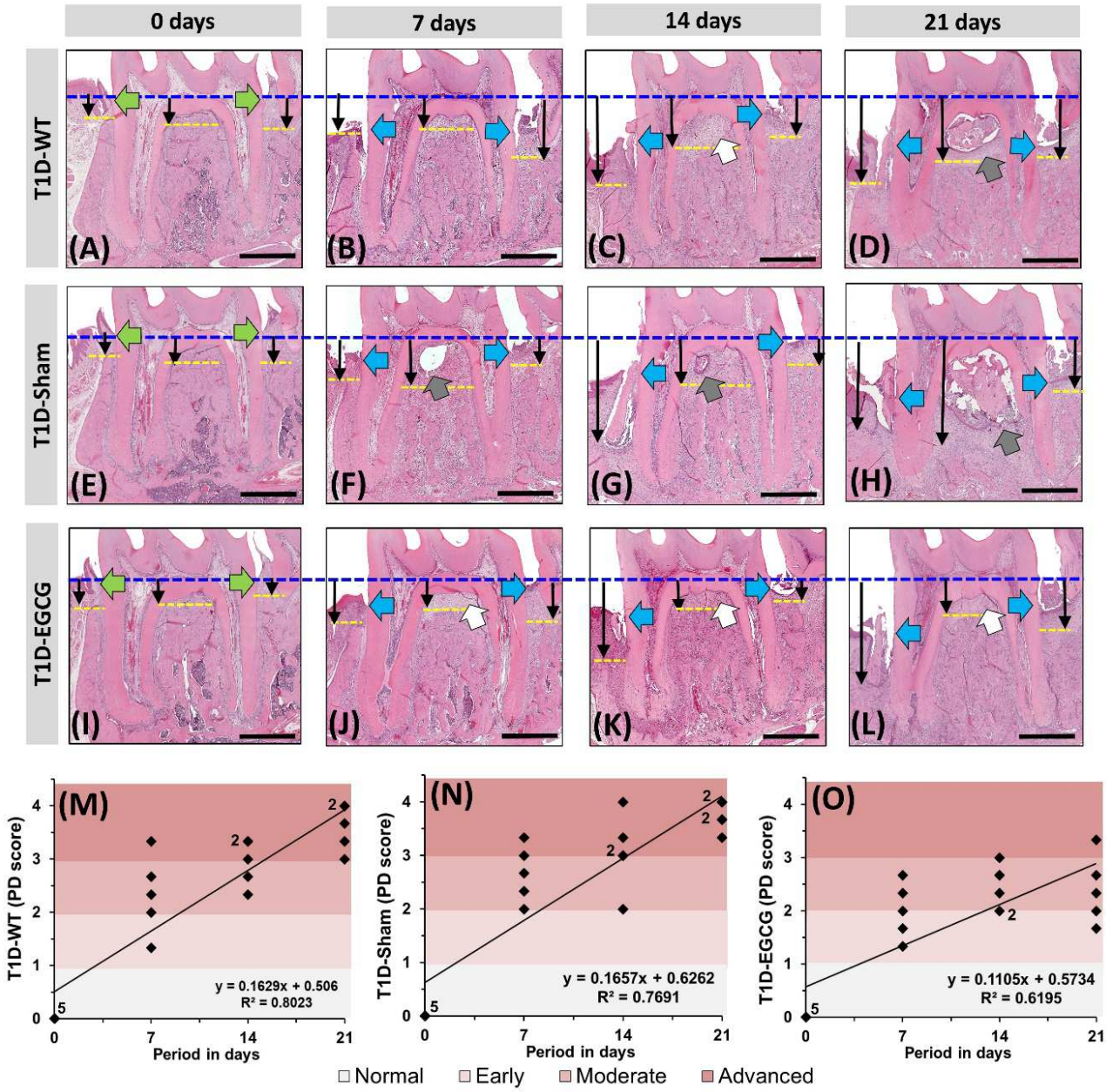

Figure 5. HE representative images (A-L) and graphic of Pearson correlation of Level of Periodontal Disease (PD-score) in M1 root of diabetic groups. HE images (A-L) and graphics of Pearson correlation of PD-score TID-WT (M), T1D-Sham (N) and T1D-EGCG (O) show a progressive periodontal breakdown during time until the end of the periods. Note advanced furcation lesions in D and H associated to greater alveolar bone loss. Gingivitis or gingival recession (blue arrow). Horizontal bone loss, alveolar bone crest or interdental septum loss until 1/3 of root (white arrow). Vertical bone loss up to 2/3 of root (grey arrow). ST Barr = 1mm.

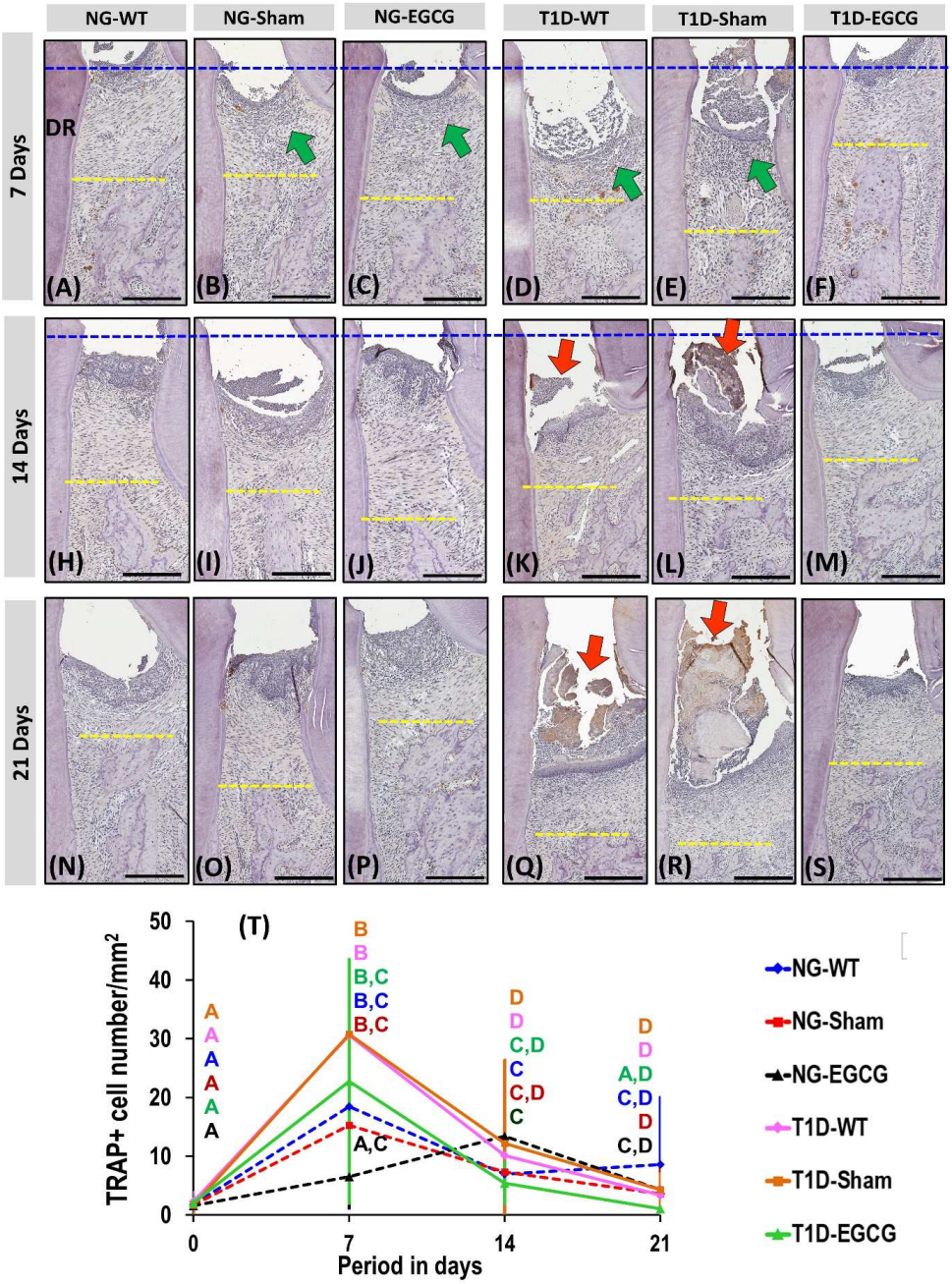

Figure 6: Histological microphotographs of counterstained slides for TRAP+ cells at 7, 14 and 21 days after induced periodontitis by ligature on the proximal region. At 7 days, in all groups, gingival and subepitelial connective tissues inflammation is evident. Additionally, an apical migration of the alveolar bone crest (yellow dashed lines) accompanied by epithelial attachment loss of the gingiva in NG-Sham, NG-EGCG, T1D-WT and T1D-Sham (green arrow) is evident. Few TRAP+ cells are also evident and they are related to root resorption at the cervical level (highlights of A, B and D). At 14 and 21 days alveolar crest apical migration and epithelial attachment loss is evident in all groups however with different epithelial and connective tissues configuration (see hihglights of H-S); T1D-WT and T1D-Sham groups display disorganized inflammatory connective tissue and loss of the epithelial insertion on root related to the presence of necrotic tissues above the epithelium (highlights of K, L, Q and R). ST bar = 300 microm (12.3x). **Graphic of counterstained slides for TRAP+ cells (T):** TRAP+ cell peak at 7 dyas, especially in T1D-WT and T1D-WT. T1D-EGCG does not present significant statistical differences between normoglicemic groups.

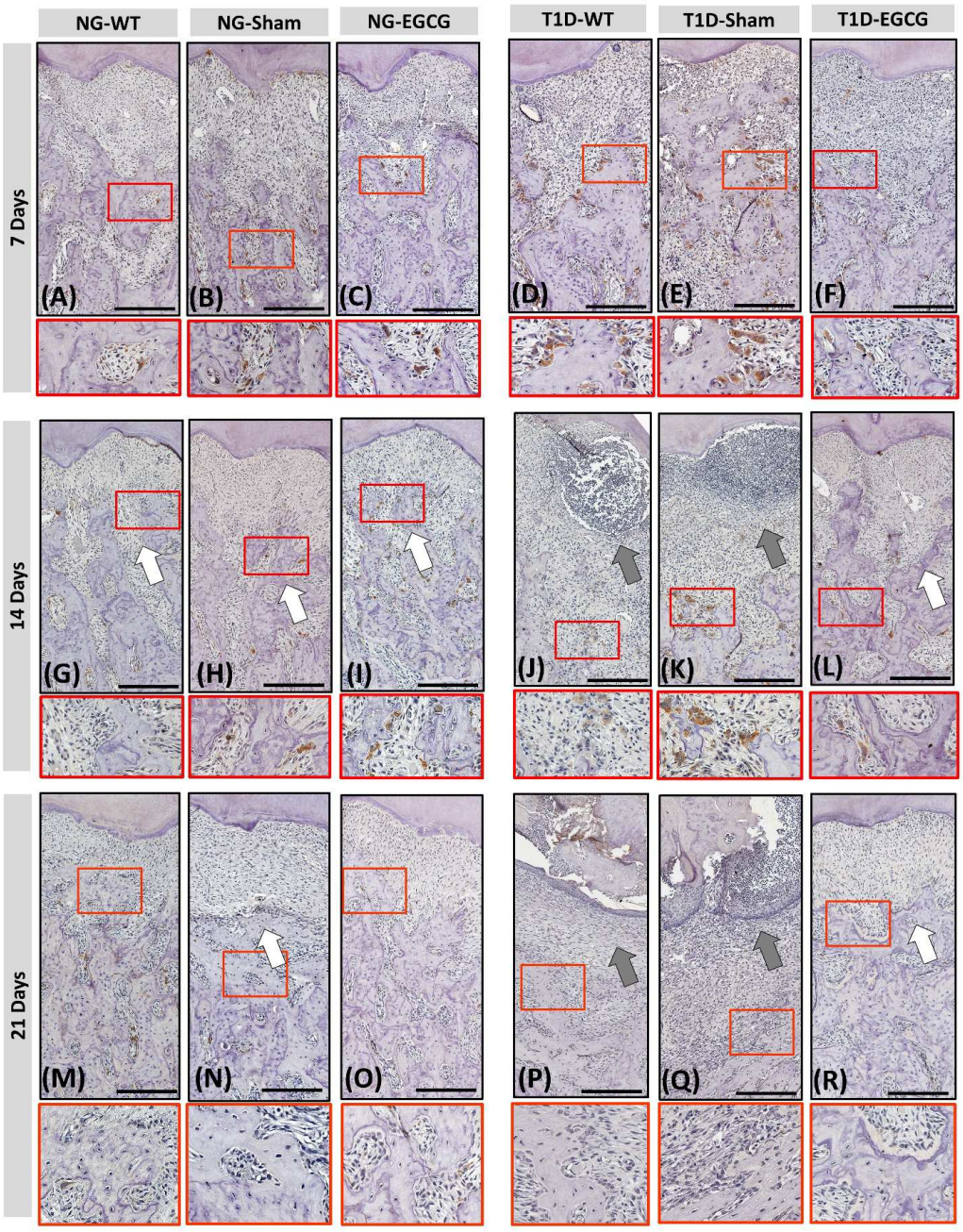

Figure 7: Histological microphotographs of counterstained slides for TRAP+ cells at 7, 14 and 21 days after induced periodontitis by ligature. On the furcation region, from 7 (A-C) to 14 days, NG-WT, NG-Sham, NG-EGCG (G-H) and T1D-EGCG (D) groups show an initial level of furcation lesion (white arrow; score 2, see figure 2) with loss of the characteristic organized periodontal fibers arrangements and substituted by disorganized inflammatory connective tissue and presence of some TRAP+ resorptive cells (red highlitghts). At 21 days (M-R) fibers organization and density of fibers start to appear and a diminution of the resorptive cells. Besides a balanced bone turnover is displayed; bone volume and reversal lines are similar among three groups, however NG-WT (M) shows less bone volume. In T1D-WT and T1D-Sham, low grade of furcation lesion is evident only at 7 days with TRAP+ cell presence (D and E). From 7 to 21 days (D, E, J, K, P and Q), progressive periodontal breackdown is observed; furcation lesions grade 2 and 3 (score 3 and 4, respectively; grey arrows), and filled by disorganized imflammatory cells and tissue however with few TRAP+ cells. Note at 21 days (P and Q) necrotic tissues are above epitelial tissue formed around interradicular alveolar bone. Besides, at 14 (J and K) and 21 days (P and Q) an unbalanced bone turnover is evident; few bone reversal lines and less volume. ST bar = 300 microm

3 DISCUSSION

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In this present thesis, we focused on the role of green tea and the polyphenol Epigallocatechin gallate (EGCG) of green tea promoting a therapeutic effect on damaged periodontal tissue in diabetic rats.

We observed that daily green tea consumption has a therapeutic effect on the diabetic vascular disorder, in periodontal ligament and the progression of periodontitis in long-term hyperglycaemia in T1D rats (article 1) and daily EGCG consumption has therapeutic effect on the periodontal disease in hyperglycemic condition, demonstrating antioxidant and anti-inflammatory actions (article 2).

In the first study (article 1), daily consumption of green tea inhibited / reduced the development of dental caries and periodontal disease in experimentally induced diabetic rats by single application of streptozotocin. The results showed a possible protective effect of green tea on oral health due to the reduction of bacterial plaque formation and pathogen virulence, besides, polyphenols present in the infusion acted systematically to reduce the inflammatory process.

It is known that the main etiological agents of dental caries and periodontal disease are produced by various restricted oral bacterial strains. Thus, most current antiplaque commercial products are composed of antimicrobials, however, many currently used bactericides and chemical antibiotics can disrupt the bacterial flora of the oral cavity, resulting in the induction and proliferation of antibiotic resistant bacteria and opportunistic pathogens (Lamster and Novak, 1992; Haffajee and Socransky, 1994).

Therefore, in recent years, polyphenols of some edible plants have attracted attention as potential sources of agents capable of controlling the growth of oral bacteria. Subsequently, in vitro studies of plant extracts suggest an activity against various metabolic active compounds of *Streptococcus mutans*, which results in decreased growth and virulence (Xiao *et al.*, 2000; Wittpahl *et al.*, 2015).

FERRAZZANO et al. (2011), suggest that daily use of green tea infused mouthwash could reduce colonization of Streptococci mutans and lactobacilli, which are the most virulent cariogenic pathogens in the oral cavity.

In the first article, we found that in the initial periods (15 days), the consumption of green tea (Camelia sinensis) as a liquid diet promoted a reduction in blood glucose. In later periods, our results corroborate with (Meneghetti, 2010), (2010) who found that, in chronic states of diabetes, the tea has no hypoglycemic effect, leading to cachexia, with reduced body mass, cataracts, polyuria and increased consumption of liquid diet.

Dental and parenteral structures in the control groups after 90 days were intact, with mild to moderate inflammatory process in the marginal gum. In the GDA group, however, extensive carious lesions with pulp necrosis, periapical abscess and severe periodontal disease led to the loss of dental and paradenteral structures. However, in the GDC group only two hemimandibles showed a pathological condition similar to that of the GDA group, and in the other eight hemimandibles, only coronal caries, inflammatory process in the pulp, partial pulp necrosis, small bone resorption and gingival recession to the level of the furcation region or cervical level.

The loss of the integrity of dental and parenteral structures in diabetics has been described in the literature in humans (FERRAZZANO et al., 2011). In experimental animal models, (GENNARO, 2012) observed that at 30 and 90 days, diabetic animals treated with green tea presented less bone loss than those treated with water. Similarly, ISHIDA et al., 2007 demonstrated that orally administration of green tea extracts are able to decrease alveolar bone resorption induced by LPS (liposaccharide) inoculation in the mouse periodontium.

At 60 days, in the Article 1, it was possible to observe the formation of dense and disorganized collagen fibres, reduction in vessel density/MVD and decrease of NcCD31+/mm^2 associated with increase of NcVEGF+/mm^2 in all PDL of T1D-W rats. Our results in the Article 1 suggest a major vascular response and healing during bacterial challenge in the diabetic periodontal tissue that received green tea. In addition, in previous study, the green tea intake reduced expression of the pro-inflammatory cytokines TNF-α and RANKL to normal levels in PDL of STZ-T1D rats

reducing the osteoclastic bone resorption (Gennaro et al., 2015) which is also observed in periodontitis induced by ligature or Escherichia coli LPS in rats treated with EGCG polyphenol-green (Cho et al., 2013; Yoshinaga et al., 2014).

The polyphenols of green tea could have also restored the secretion of antimicrobial peptides, such as beta-defensins, by gingival epithelial cells (Lombardo Bedran *et al.*, 2014) and/or improved immunomodulatory responses to bacteria by neutrophils (Lalla & Papapanou, 2011).

Inhibited cytokine expression, including TNF and IL-6 are responsible for the reduction in osteoclast formation, osteoclastic activity and collagen destruction (Cho *et al.*, 2013). In the article 2 we found that systemic administration of EGCG could have a therapeutic effect on periodontal tissue damage, but in diabetic rats this same analyses are not yet found in the literature.

In the results after ligature, the total PBL showed a gradual increase in all experimental groups until 14 days ($p>0.05$). But, at 21 days the values maintained similar to that of 14 days ($p>0.05$) in the NG-WT (972±167μm), NG-Sham (1120±118μm), NG-EGCG (968±153μm) and T1D-EGCG (1087±122μm), while in the T1D-WT and T1D-Sham the total PBL increased in mean 132% ($P<0.01$). Besides that, BV/TV reduction was observed in all experimental groups after ligature, however in normoglycemic groups and T1D-EGCG, this BV/TV reduction was with no statistical differences. These results can be explained by the regulative effect on osteogenic function of EGCG, that probably affects osteogenetic differentiation through the modulation of BMP-2 expression. Moreover, EGCG has been found to improve cell growth, a finding that might be related to its potential as an antioxidant producing favorable amounts of ROS in the cellular environment (Jin *et al.*, 2014).

Previous studies have shown that the ratio of *P. gingivalis* was higher than other oral bacteria in chronic periodontitis, and that it invaded periodontal tissue to increase the expressions of inflammatory mediators, especially interleukins (IL)-1β, IL-6, IL-17 and tumor necrosis factor TNF-α. Several studies have also shown that IL-6 and TNF-α are associated with periodontitis in vivo and in vitro, and are down-regulated by EGCG treatment (Cai *et al.*, 2015). The level of Periodontal Disease (PDL), after ligature alterations in the periodontal structures, such as inflammatory

process, pocket's epithelium, destruction of the collagenous periodontal ligament and bone resorption were observed in all experimental groups (Siggelkow *et al.*, 2003; Cho *et al.*, 2013). The PD-scores in T1D-EGCG did not show statistical differences when compared to NG groups, evidencing the protective activity of EGCG. In a previous work, a EGCG-treated group showed significant decreased expression of IL-1β, IL-6, TNF-α, RANKL, CCL2 and MMP-9 in the gingival tissue (Cai *et al.*, 2015). This is one of the possible ways of action of EGCG for improving periodontal disease in diabetic animals with ligature-induced periodontal disease.

4 CONCLUSIONS

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In conclusion, this study originally demonstrated

- Green tea *ad libittum* improves glycemic control (Article 1), and can be used as a possible therapy adjunct to mechanical oral hygiene procedures in diabetic patients who have a risk of poor glycemic control. Different from aerticle 2 in which EGCG was used as a treatment in diabetic animals (Article 2).

- Green tea is capable of promoting tissue vascularization decreasing the dental plaque accumulation and periodontal tissue loss in long-term of hyperglycaemia in T1D rats (Article 1).

- Systemic administration of EGCG may influence the host inflammatory immune response improving the periodontal morphology. In addition, EGCG could have a therapeutic effect through inhibition of inflammatory cytokines, in response to the reduction in osteoblast formation, osteoclastic activity and collagen destruction. EGCG can be used as an auxiliary therapeutic agent in periodontal disease in diabetic patients (Article 2).

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APPENDIXES

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ANNEXES

ANNEX

Annex 1: Authorization of the publisher when article accepted for publication

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Anenx 2: Approval of Animal Ethical Committee

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

50 ANOS 1962 - 2012 FOB USP

CEEPA-Proc. Nº 032/2013

Bauru, 20 de agosto de 2013.

Senhor Professor,

O projeto de pesquisa encaminhado a esta Comissão de Ética no Ensino e Pesquisa em Animais, denominado **Efeito da administração oral da epigalocatequina-3-galato durante a periodontite induzida por ligadura em ratos diabéticos**, de autoria de Daniela Santos Pereira, com colaboração de Tania Mary Cestari e Ever Elias Mena Laura, sob sua orientação, foi enviado a um relator para avaliação e considerado APROVADO em reunião desta Comissão, realizada no dia **19 de agosto de 2013**.

Solicitamos que qualquer alteração na pesquisa seja comunicada a esta Comissão, e que, ao final seja enviado um Relatório com os resultados obtidos, para análise ética e emissão de parecer final, o qual poderá ser utilizado para fins de publicação científica.

Atenciosamente,

Prof. Dr. Gustavo Pompermaier Garlet
Presidente da Comissão de Ética no Ensino e Pesquisa em Animais

Prof. Dr. Gerson Francisco de Assis
Docente do Departamento de Ciências Biológicas

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Anenx 3: Description of the product used as a therapeutic treatment in article 2 (EGCG)

Sunphenon® EGCg

Specialized Green Tea Extract Powder

Date: March 1, 2011

Sunphenon® EGCg is a decaffeinated extract of green tea leaves (*Camellia sinensis*), made of highly purified natural green tea catechins, rich in epigallocatechin gallate (EGCg). Sunphenon® EGCg has minimal coloring with little to no taste, ideal for use in antioxidant rich supplements, beverages, dairy products, confections and foods.

Sunphenon® EGCg is Food Grade, non GMO and certified Ⓚ Kosher.
Sunphenon® EGCg complies with U.S. FDA pesticide regulations for tea as outlined in 40CFR180.

Specifications

Item	Specification Value	Method / Condition
Appearance	Off-white to pale-pink powder	Visual Observation
EGCg content	Not less than 94%	HPLC, Dry Matter*
Caffeine	Less than 0.1%	HPLC
Loss on Drying	Less than 5.0%	105˚C, 3 hours
Residue on Ignition	Less than 0.5%	550˚C, 3 hours
Bulk Density	0.42 ~ 0.62 g/ml	LBD
Heavy Metals (as Pb)	Less than 10.0 μg/g	Colorimetry
Arsenic (as As_2O_3)	Less than 1.0 μg/g	Atomic-photospectrometry
Arsenic (as As)	Less than 1.0 μg/g	Atomic-photospectrometry
Lead (Pb)	Less than 1.0 μg/g	Atomic-photospectrometry
Cadmium (Cd)	Less than 0.5 μg/g	Atomic-photospectrometry
Mercury (Hg)	Less than 0.1 μg/g	Atomic-photospectrometry
Standard plate count	Less than 1,000 cfu/g	Standard Plate Agar
Coliforms	Negative / 0.1g	BGLB method
E. coli	Negative / 0.1g	BGLB method
Mold / Yeast	Less than 100 cfu/g	Potato dextrose agar plate / Chloramphenicol
Salmonella	Negative / 25g	SMAFSRB**
Staphylococcus aureus	Negative / g	SMAFSRB**

* Dry Matter

** SMAFSRB: Standard Methods of Analysis in Food Safety Regulation Biology, Japan.

Packaging and Storage

10 kg net weight, aluminum foil bag, carton drum. In its original packaging, may be stored at room temperature at least 36 months from date of production. Store in a cool, dry place away from heat and direct light.

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