UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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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.
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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.

4 CONCLUSIONS

4 CONCLUSIONS

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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ANNEXES

ANNEX

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

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Anenx 2: Approval of Animal Ethical Committee

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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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