FABIANA FURTADO FREITAS

EVALUATION OF THE EFFECT OF TOOTH MOVEMENT ON THE DEVELOPMENT OF DIABETES-INDUCED NEUROPATHY IN RATS

AVALIAÇÃO DO EFEITO DA MOVIMENTAÇÃO ORTODÔNTICA NO DESENVOLVIMENTO DE NEUROPATIA DECORRENTE DO DIABETES INDUZIDO EM RATOS

PIRACICABA
2014

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Dissertation presents to the Piracicaba Dentistry School of the University of Campinas in partial fulfillment of the requirements for the degree of Master Dentistry, in Pediatric Dentistry area.

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Orientadora: Profª. Drª. Juliana Trindade Clemente Napimoga

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ABSTRACT

Diabetes is known to result in a greater inflammatory response that in turn accentuated orthodontic tooth movement. Thus, this study aimed to evaluate if the higher inflammatory response induced by the orthodontic tooth movement in diabetic animals changes the neuronal excitability in the trigeminal ganglia. For that, Wistar rats (± 150 g, n=4-6/group) were treated with an intraperitoneal injection of citrate buffer (vehicle; Normoglycemic – NG), streptozotocin 75 mg/kg (Diabetic – DG) or streptozotocin 75 mg/kg + subcutaneous injection of insulin (Diabetic treated with insulin – IG). Twenty-eight days after the treatment, an orthodontic appliance was placed and the tooth movement was evaluated at days 0, 1, 3, 6 and 12. After the corresponding time, the animals were terminally anesthetized and their maxillae, trigeminal ganglia and gingival tissue were removed and submitted to analyze the amount of tooth movement and biochemical analysis (ELISA) to measure the release of glutamate, Tumor Necrose Factor-alpha (TNF-α), Interleukin 1-beta (IL-1β), Substance P (SP) and Calcitonin Gene-Related Peptide (CGRP). The results demonstrated that diabetes accentuated orthodontic tooth movement at days 1, 3 and 6 when compared with NG and IG ($p<0.05$: Two-way ANOVA, Bonferroni's test). Corroborating these results, DG rats demonstrated higher release of TNF-α and IL-1β than that observed for the NG and IG rats ($p<0.05$). Although the greater inflammatory response induced in DG rats, the release of glutamate, SP and CGRP were significantly reduced ($p<0.05$). The results suggest that neuronal activation in trigeminal ganglia is reduced in diabetes.

Keywords: Diabetes Mellitus, Experimental; Tooth Movement, Inflammation, Neural Conduction.

RESUMO

O diabetes induz resposta inflamatória acentuada resultando em maior movimentação dental por dispositivos ortodônticos. Sendo assim, este estudo teve como objetivo avaliar se o aumento da resposta inflamatória, decorrente do tracionamento dental por um dispositivo ortodôntico, induzido pelo diabetes altera a excitabilidade neuronal no gânglio trigeminal. Para este estudo foram utilizados ratos Wistar (±150 g, n= 4-6/grupo) tratados com injeção intraperitoneal de tampão citrato (veículo; Normoglicêmicos – NG), estreptozotocina 75 mg/kg (Diabético – DG), ou estreptozotocina 75 mg/kg + injeção subcutânea de insulina (Diabético tratado com insulina – IG). Vinte e oito dias após o tratamento foi instalado um dispositivo ortodôntico e a movimentação dentária foi avaliada nos dias 0, 1, 3, 6 ou 12. Após o tempo correspondente, os animais foram anestesiados e a maxila, gânglio trigeminal e tecido gengival removidos e submetidos à análise para quantificação da movimentação dentária e análise bioquímica (ELISA) para avaliação da liberação de glutamato, Fator de Necrose Tumoral-alfa (TNF-α), Interleucina 1-beta (IL-1β), Substância P (SP) e Peptídeo Relacionado ao Gene da Calcitonina (CGRP). Os resultados demonstraram que o diabetes aumentou significativamente o movimento dental induzido pelo tracionamento ortodôntico nos dias 1, 3 e 6 quando comparado aos animais NG e IG ($p<0.05$: Two-way ANOVA, Teste de Bonferroni). Corroborando com esses resultados, os animais DG demonstraram maior liberação de TNF-α e IL-1β no tecido gengival em relação aos animais NG e IG ($p<0.05$). No entanto, apesar do acentuado processo inflamatório nos animais DG, a liberação de glutamato (gânglio trigeminal), SP e CGRP (tecido gengival) foi significativamente reduzida ($p<0.05$). Os resultados sugerem que a ativação neuronal no gânglio trigeminal é reduzida no diabetes.

Palavras-chave: Diabetes Mellitus Experimental, Movimentação Dentária, Inflamação, Condução Nervosa.

SUMÁRIO

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"Mesmo quando tudo parece desabar, cabe a mim decidir entre rir ou chorar, ir ou ficar, desistir ou lutar; porque descobri, no caminho incerto da vida, que o mais importante é o decidir."

Cora Coralina

LISTA DE ABREVIATURAS E SIGLAS

AMPA: alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico

CGRP: Peptídeo Relacionado ao Gene da Calcitonina

DG: Grupo de animais Diabéticos

GABA: Ácido gama-aminobutírico

IG: Grupo de animais diabéticos tratados com Insulina

IL-1β: Interleucina-1beta

IL-6: Interleucina-6

IL-8: Interleucina-8

KC: Quimiocina derivada de queratinócito

NG: Grupo de animais Normoglicêmicos

NMDA: N-metil-D-aspartato

SP: Substância P

TNF-α: Fator de Necrose Tumoral-alfa

INTRODUÇÃO

Diabetes Mellitus é uma doença metabólica caracterizada por aumento anormal de glicose no sangue como resultado de alterações na produção de insulina ou na resposta dos tecidos à insulina - hormônio pancreático responsável pela absorção de glicose no organismo e posterior utilização da mesma para produção de energia (Alberti & Zimmet, 1998). Segundo a Associação Americana de Diabetes e a Organização Mundial de Saúde (OMS) o diabetes pode ser classificado como: diabetes mellitus tipo 1 (insulinopênico), tipo 2 (resistência à insulina ou insulinopenia relativa), gestacional e outros tipos específicos de diabetes mellitus.

Apesar do diabetes, principalmente tipos 1 e 2, apresentarem etiologias diferentes, compartilham sintomas comuns, como por exemplo, intolerância à glicose, hiperglicemia e hiperlipidemia, consideradas condições crônicas que requerem monitoramento e controle cuidadosos. Além do mais, os dois tipos de diabetes apresentam complicações similares, dentre elas, anormalidades vasculares e neuropatias periféricas sensoriais (Archer *et al.*, 1983; Singleton *et al.*, 2003; Boulton *et al.*, 2004; Tesfaye *et al.*, 2010; Tesfaye & Selvarajah, 2012; Gilbert, 2013; Fatehi *et al.*, 2013).

Especificamente, em relação às neuropatias periféricas sensoriais induzidas pelo diabetes, estas representam complicações debilitantes afetando mais de 50% dos pacientes diabéticos (Boulton *et al.*, 2004). O desenvolvimento das neuropatias periféricas sensoriais induzidas pelo diabetes está diretamente associado com a falta do controle glicêmico, tempo de instalação da doença, fatores microvasculares (microangiopatias) e fatores de risco cardiovasculares (hipertensão, hiperlipidemia, obesidade e fumo) (Tesfaye *et al.*, 1996; Boulton *et al.*, 2004; Tesfaye & Selvarajah, 2012). São descritas como distúrbios no sistema nervoso periférico, ocasionados por alterações estruturais nas fibras neuronais – como rompimento das células de Schwann (desmielinização), degeneração e perdas axonais, lesões microvasculares e alterações nas sinalizações bioquímicas intracelulares (Arezzo & Zotova, 2002). Estas neuropatias induzem a uma variedade de alterações na condução nervosa incluindo dor espontânea, hiperalgesia e alodínea, assim como quadros de hipoalgesia e analgesia (Calcutt, 2004).

Neste sentido, a principal queixa de pacientes diabéticos é, frequentemente, perda de sensibilidade nas extremidades (hipoalgesia), que em estágios avançados pode evoluir para completa analgesia (ausência quase total dos nervos periféricos), facilitando a ocorrência de acidentes que acarretam em danos teciduais e quadros infecciosos, podendo levar, em casos mais graves, à amputação do membro afetado (Calcutt, 2004). Apesar da literatura escassa, estudos em humanos tem sugerido que as alterações decorrentes do diabetes também estão relacionadas com distúrbios do sistema estomatognático, tais como perda precoce de elementos dentais, disfunções temporomandibulares, dor orofacial, síndrome da ardência bucal e periodontite (Collin *et al.*, 2000; Arap *et al*., 2010; Zhu & Nilolajczyk, 2014). Neste sentido, um estudo em animais demonstrou que o diabetes tipo 1 aumenta significativamente a reabsorção óssea durante a movimentação ortodôntica como resultado de uma potencialização do processo inflamatório (Braga *et al.,* 2011).

A movimentação dentária induzida por aplicação de força ortodôntica é caracterizada por alterações na remodelação dos tecidos dentais e periodontais, como osso alveolar, polpa dental, ligamento periodontal e gengiva (Krishnan & Davidovitch, 2006). A força aplicada provoca compressão e reabsorção do osso alveolar e ligamento periodontal de um lado, enquanto no lado oposto ocorre estiramento do ligamento e aposição óssea (Dolce *et al.*, 2002). O osso alveolar e tecidos periodontais adjacentes, quando expostos a diferentes graus de magnitude, frequência e duração de carga mecânica, mostram grandes alterações macroscópicas e microscópicas. Esta carga mecânica altera a vascularização do tecido periodontal e o fluxo sanguíneo, o que resulta na síntese e liberação de diversas moléculas, como neurotransmissores, citocinas proinflamatórias, fatores de crescimento, fatores estimuladores de colônias e metabólitos do ácido araquidônico (Davidovitch *et al.*, 1988; Davidovitch, 1991). Como consequência da liberação destes mediadores químicos, um efeito comum do tratamento ortodôntico é a dor, relatada pelos pacientes como sendo impedimento para a realização do mesmo e um dos principais motivos para a interrupção do tratamento (Haynes, 1974; Oliver & Knapman, 1985; Brown & Moerenhout, 1991; Kluemper *et al.*, 2002).

É bem estabelecido na literatura que a dor é um dos sinais clássicos de processo inflamatório instalado, apresentando como denominador comum, a sensibilização dos

receptores nociceptivos aferentes primários. Decorrente de estímulos inflamatórios ou lesões teciduais, a liberação de citocinas e quimiocinas pró e anti-inflamatórias desencadeia a liberação de prostanoides e aminas simpatomiméticas que, por sua vez, atuam diretamente em nociceptores causando hipernocicepção, resultado da redução do limiar de excitabilidade devido à modulação de canais de sódio voltagem-dependentes (Khasar *et al.*, 1998, Verri *et al.*, 2006; Gold & Gebhart; 2010). Neste sentido, foi proposto que o TNF-α (Fator de Necrose Tumoral-alfa), uma das principais citocinas proinflamatórias, induz hipernocicepção através de duas vias paralelas e independentes: (1) via IL-1β/IL-6 (Interleucina-1beta/Interleucina-6) por meio de prostanoides; e (2) via IL-8/KC (Interleucina-8/Quimiocina derivada de queratinócito) por meio de aminas simpatomiméticas (Cunha *et al.*, 2005; Verri *et al.*, 2006).

Esta hiperexcitabilidade dos neurônios aferentes primários é o principal fator para o desenvolvimento e manutenção de neuropatias periféricas (Ji & Strichartz, 2004; Rho & Prescott, 2012; Tsantoulas *et al.*, 2012; Crown *et al.*, 2012), como resultado do aumento da liberação de neurotransmissores excitatórios (glutamato) e neuropeptídeos (substância P e Peptídeo Relacionado ao Gene da Calcitonina) no sistema nervoso central (Skilling *et al.*, 1992; Mark *et al.*, 1998; Gardell *et al.*, 2003; Coderre *et al.*, 2005; Li *et al.*, 2010; Doolen *et al.*, 2012; Calcutt, 2013; Orestes *et al.*, 2013; Osikowicz *et al.*, 2013; Yan *et al.*, 2013).

Sendo assim, considerando que o diabetes tipo 1 aumenta significativamente a movimentação dental por tracionamento ortodôntico como resultado de um processo inflamatório acentuado (Braga *et al*., 2011); é possível que esta exacerbação do processo inflamatório induzido pelo diabetes altere a atividade neuronal do sistema trigeminal induzindo um quadro de neuropatia periférica diabética neste sistema. Mudanças no comportamento e estilo de vida dos seres humanos, como sedentarismo e obesidade, no último século, têm resultado em um dramático aumento na incidência do diabetes no mundo (Zimmet *et al.*, 2001). Segundo dados da OMS de 2012, um em cada dez adultos tem diabetes mellitus. De acordo com a Federação Internacional do Diabetes, 285 milhões de pessoas no mundo têm a doença e estima-se que para o ano de 2030 este número chegue a 438 milhões de pessoas, tratando-se de um crescente problema de saúde pública. Dados

estes alarmantes, tendo em vista que as complicações da doença causam um alto índice de morbidade e mortalidade (Libman *et al.*, 1993; Lin *et al.*, 2014).

Diante do exposto, consideramos que o trabalho proposto é de relevância clínica uma vez que auxilia no melhor entendimento sobre o efeito do diabetes na resposta inflamatória e alterações sensoriais induzidas pelo tratamento ortodôntico.

* Esta dissertação está apresentada em formato alternativo, conforme deliberação da Comissão Central de Pós-Graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) nº 228/2013.

CAPÍTULO 1

Diabetes reduces neuronal activation during orthodontic tooth movement

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Abstract

Diabetes in early phase is known to result in a greater inflammatory response that in turn accentuated orthodontic tooth movement. Thus, this study aimed to evaluate if the higher inflammatory response induced by the orthodontic tooth movement in diabetic animals changes the neuronal excitability in the trigeminal ganglia. For that, Wistar rats (± 150 g, n=4-6/group) were treated with an intraperitoneal injection of citrate buffer (vehicle; Normoglycemic – NG), streptozotocin 75 mg/kg (Diabetic – DG) or streptozotocin 75 mg/kg + subcutaneous injection of insulin (Diabetic treated with insulin – IG). Twenty-eight days after the treatment, an orthodontic appliance was placed and the tooth movement was evaluated at days 0, 1, 3, 6 and 12. After the corresponding time, the animals were terminally anesthetized and their maxillae, trigeminal ganglia and gingival tissue were removed and submitted to analyze the amount of tooth movement and biochemical analysis (ELISA) to measure the release of glutamate, Tumor Necrose Factor-alpha (TNF-α), Interleukin 1-beta (IL-1β), Substance P (SP) and Calcitonin Gene-Related Peptide (CGRP). The results demonstrated that diabetes accentuated orthodontic tooth movement at days 1, 3 and 6 when compared with NG and IG ($p<0.05$; Two-way ANOVA, Bonferroni's test). Corroborating these results, DG rats demonstrated higher release of TNF-α and IL-1β than that observed for the NG and IG rats ($p<0.05$). Although the greater inflammatory response in DG rats, the release of glutamate, SP and CGRP were significantly reduced ($p<0.05$). The results suggest that neuronal activation in trigeminal ganglia is reduced in the early phase of diabetes.

Keywords: diabetes, tooth orthodontic movement, inflammation, neuronal activation.

Introduction

Diabetes mellitus affects over 285 million people worldwide and according to International Diabetes Federation (IDF) there is a prediction that in 2030 it will be around 428 million. The disease has a dramatic impact on health and its complications cause a high degree of morbidity and mortality (Libman *et al.*, 1993; Lin *et al*., 2014). It includes several complications like vascular abnormalities, peripheral sensory neuropathy, increased susceptibility to infection and greater risk of periodontal disease (Löe, 1993; Tesfaye and Selvarajah, 2012; Gilbert, 2013).

Particularly, diabetic peripheral neuropathies includes a variety of painful conditions, including since spontaneous pain, hyperalgesia and allodynia to hipoalgesia and analgesia (Calcutt, 2004; Tesfaye and Selvarajah, 2012), as a result of modifications in the neuronal activation and transmission. The main complaint of diabetic patients is often loss of sensation in the extremities (hypoalgesia), which in advanced stages may progress to complete analgesia (total absence of peripheral nerves) (Calcutt, 2004). The mechanism underlying peripheral neuropathies induced by diabetes is not completely understood, but it is associated with increased release of excitatory neurotransmitters and neuropeptides in the central nervous system (Calcutt, 2013; Osikowicz *et al.*, 2013).

Considering the complications of diabetes in the oral system, an animal study demonstrated that diabetes in mice induced a higher orthodontic tooth movement as a result of an augmented levels of pro-inflammatory mediators, such as Factor Necrosis Tumor - alpha (TNF-α) and alveolar bone turnover (Braga *et al.*, 2011). Tooth movement induced by orthodontic force application is characterized by remodeling changes in the dental and periodontal tissues, like alveolar bone, dental pulp, periodontal ligament and gingiva (Krishnan and Davidovitch, 2006). In addition, mechanical loading also alters periodontal tissue vascularity and blood flow, resulting in local synthesis and release of neurotransmitters and cytokines (Krishnan and Davidovitch, 2006). As a result of the release of chemicals mediators, one extremely negative effect of orthodontic treatment is the pain, which is reported by the patients as a major reason for discontinuing treatment or

even being impediment to its realization (Brown and Moerenhout, 1991; Kluemper *et al.*, 2002).

It is well been established that the release of inflammatory cytokines and chemokines triggers the release of prostanoids and sympathetic amines that act directly on nociceptors causing hypernociception (Khasar *et al.*, 1998, Verri *et al.*, 2006).

Thus, considering that diabetes is associated with a higher production of inflammatory cytokines, which in turn, stimulate a persistent stimulus for leukocyte recruitment resulting in a maintenance and amplification of inflammation (Naguib *et al.*, 2004; Graves *et al.*, 2005); the aim of this study was to evaluate if the accentuated inflammatory response induced by diabetes, that result in a greater orthodontic tooth movement, cause changes into the neuronal excitability in the trigeminal ganglia.

Material and Methods

Animals

This study was carried out with male Wistar rats obtained from the Multidisciplinary Center for Biological Research (CEMIB) at the University of Campinas (Campinas, São Paulo, Brazil) (200-250 g) maintained in a temperature-controlled room ($23^{o} \pm 1^{o}C$) with a 12 hour light/dark cycle. They were housed four per cage with food and water *ad libitum.* It was used crumbled diet. All animal experimental procedures and protocols were approved by the Committee on Animal Research of the University of Campinas (#2559-1) and are in accordance with guidelines of National Council for Control of Animal Experimentation (CONCEA) and International Association for the Study of Pain (IASP) guidelines for the study of pain in conscious animals (Zimmermann, 1983). The animals suffering and number per group (5 per group) were kept at a minimum and each animal was used once.

Induction of diabetes and experimental design

The protocol used to induce diabetes was performed as previously described (Courteix *et al.*, 2007). The animals received an intraperitoneal injection of 75 mg/kg of Streptozotocin (STZ®; Sigma-Aldrich, USA) freshly dissolved in 0.1M of citrate buffer (pH 4.5). The rats were fasted for 8 h prior to STZ injection. The induction of diabetes was confirmed seven days later by measurement of plasma glucose levels of blood samples collected from the tail vein using the glucose-oxidase enzymatic method (Optium Xceed; Abbott®). Only animals with blood glucose concentration greater than 300 mg/dl after 8 h of fasting were included in the study (Braga *et al.*, 2011). Experiments were conducted in three different animals groups: (1) Diabetics animals group (DG) treated with STZ; (2) Normoglycemic animals group (NG) treated with citrate buffer (vehicle); and (3) Diabetics animals group treated with Insulin (IG) (confirmed three days after the STZ administration) treated with daily subcutaneous injections of Insulin (3 U/100 g body weight of Humulin® N and Humulin® R, Lilly, USA – Suthagar *et al.*, 2009) twice a day - 9:00 and 21:00 h. Weight and plasma glucose concentration were recorded once a week during the experimental period. Thirty days after the initiation, an orthodontic appliance was placed and tooth movement was evaluated after 0, 1, 3, 6 or 12 days. Immediately after the evaluation of orthodontic tooth movement, the animals were terminally anesthetized and their maxillae, trigeminal ganglia and gingival tissue were removed for further analysis. Maxillae were used to evaluate the orthodontic tooth movement and trigeminal ganglia and gingival tissue were used to evaluate the release of inflammatory cytokines (TNF-α and IL-1β), the neurotransmitter glutamate and neuropeptides (SP and CGRP).

Orthodontic appliance placement and measurement of tooth movement

The orthodontic appliance placement used was adapted from Gameiro *et al.* (2008). The animals were anesthetized with xylazine (10 mg/kg) and ketamine (50 mg/kg), and a closed coil nickel-titanium spring (Morelli®, Campinas, Brazil), calibrated to provide

a force of 50 g, was connected to the upper left first molar and connected to incisors by ligature metal and light-cured resin.

After experimental period, animals were killed and the maxillae were removed and fixed in 10% buffered formalin. Maxillae were stained with methylene blue and then photographs were taken by digital camera Canon EOS Rebel T3i for quantification of tooth movement using Image J software (National Institutes of Health, USA). The distance between the mesial surface of the first molar and the distal surface of the third molar was measured bilaterally by a calibrated person who was blinded to the experimental conditions. Tooth movement was estimated by subtracting the average of the values obtained by repeated measurements of the treated and untreated sides (Gameiro *et al.*, 2008).

Enzyme-linked immunosorbent assay (ELISA)

Trigeminal ganglia and gingival tissues were homogenized in 500 µl of appropriate buffer containing protease inhibitors (Ripa Lysis Buffer®, Santa Cruz, Biothecnology, Dallas, Texas, USA) followed by a centrifugation of 10 min/10.000 rpm/ 4 ºC). The levels of glutamate (Abcam®, Cambridge, MA, USA), substance P (Phoenix Pharmaceuticals®, Inc., Burlingame, California, EUA), calcitonin gene-related peptide (CGRP) (Phoenix Pharmaceuticals®, Inc., Burlingame, California, EUA), TNF-α and IL-1β (RD Systems®, Minneapolis, MN, USA) were determined by capture enzyme-linked immunosorbent assays (ELISA) using protocols supplied by the manufacturer.

Statistical analyses

Data were analyzed using two-way analysis of variance (Two-way ANOVA) with two between factors (group x time) was used to determine if there were significant differences in tooth movement or mediators release among the groups. When statistically significant differences were identified, individual comparisons were subsequently made using Bonferroni's test. Statistical significance was set at $p<0.05$. The software GraphPad

Prism 4.0 (La Jolla, CA, USA) was used to perform statistical calculations. Data are reported as means ± SD.

Results

The diabetic group (DG) showed orthodontic tooth movement greater than that observed in normoglycemic group (NG) and diabetic + insulin group (IG) after 0, 1, 3 and 6 days of orthodontic appliance placement ($p<0.05$; Two-Way ANOVA, Bonferroni′s Test) (Figure 1). The IG group demonstrated a significant lower orthodontic tooth movement than that observed in NG group at day 6 ($p<0.05$). These results suggest that diabetes augmented tooth movement induced by orthodontic appliance.

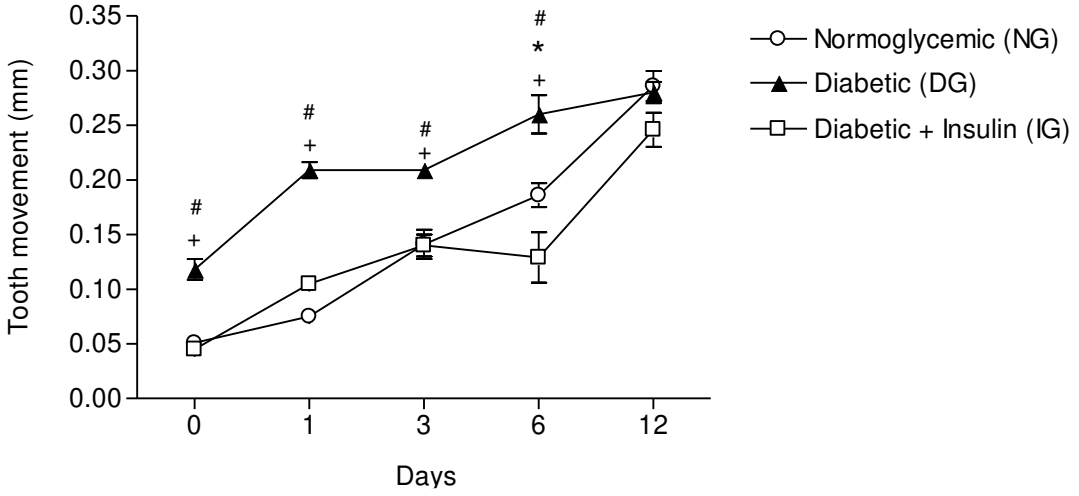

Figure 1. **Diabetes augments tooth movement induced by orthodontic appliance**. The DG showed orthodontic tooth movement greater than that observed in NG and IG times 0, 1, 3, and 6 and greater than that observed in IG at times 0, 1 and 6 days. The IG showed lower orthodontic tooth movement than that observed in NG only at day 6 ($p<0.05$: Two-way ANOVA, Bonferroni's test). The symbol (+) indicates statistical difference between DG and NG groups. The symbol (*) indicates statistical difference between NG and IG groups. The symbol (#) indicates statistical difference between DG and IG groups.

Considering that the diabetes increased the orthodontic tooth movement as a result of an augmented of inflammatory response, it was evaluated the release of inflammatory cytokines TNF-α and IL-1β in the gingival tissue. Corroborating the first set of experiments, diabetes induced a release of TNF-α significantly higher than that observed in the NG and IG animals ($p<0.05$; Two-way ANOVA, Bonferroni′s Test) (Figure 2A). The results demonstrated that the release of IL-1β was significantly higher than that NG and IG at day 1, 3 and 6 after orthodontic appliance placement ($p<0.05$) (Figure 2B). It was observed that IG released IL-1β significantly lower than that NG at day 1 ($p<0.05$) (Figure 2B).

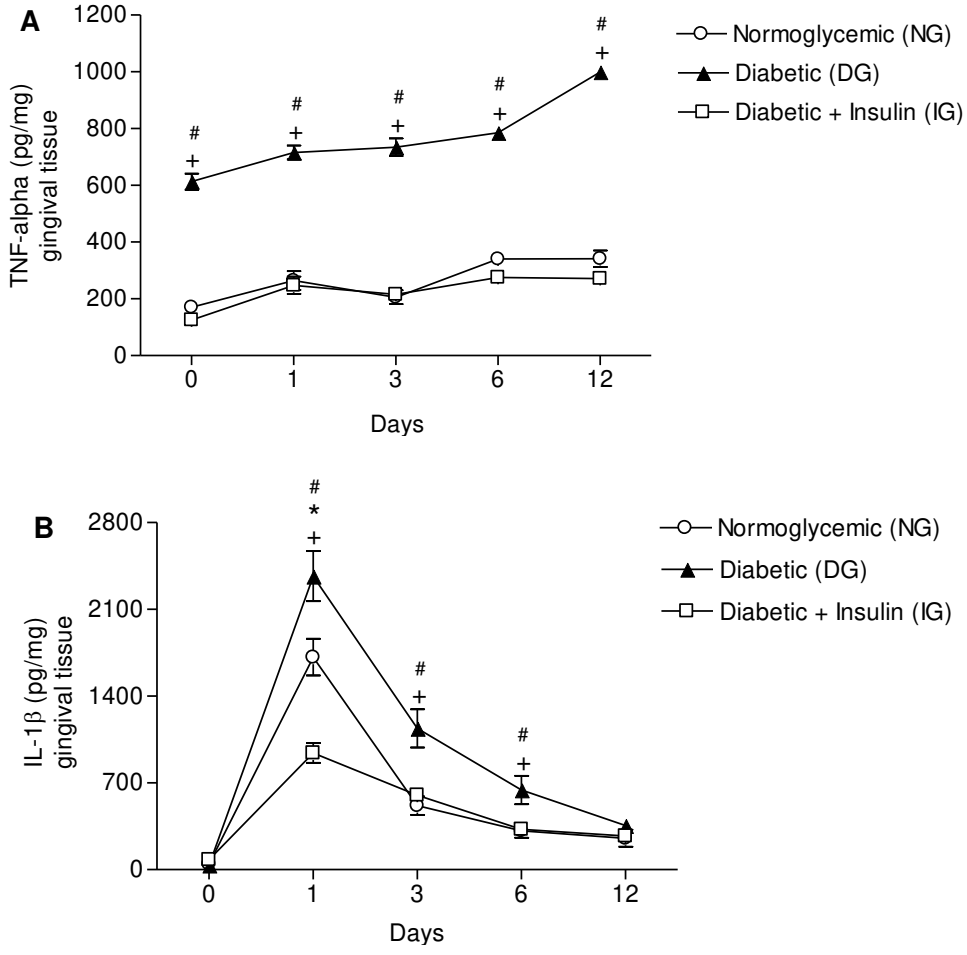

Figure 2. **Diabetes increases the release of inflammatory cytokines in the gingival tissue. (A)** The release of TNF-α in the gingival tissue was significantly higher in DG when compared to that NG and IG

($p<0.05$; Two-way ANOVA, Bonferroni's test). There was no statistical difference between NG and IG ($p>0.05$). **(B)** The release of IL-1β in the gingival tissue was significantly higher in DG when compared to that NG and IG at times days 1, 3 and 6 ($p<0.05$; Two-way ANOVA, Bonferroni's test). The release of IL-1β was significantly lower in IG than that NG at day 1. The symbol (+) indicates statistical difference between DG and NG groups. The symbol (*) indicates statistical difference between NG and IG groups. The symbol (#) indicates statistical difference between DG and IG groups.

To evaluate if the elevated inflammatory response induced by diabetes as a result of orthodontic tooth movement may increase the release of excitatory neurotransmitter in the trigeminal ganglia, it was measured the release of the glutamate (a classical excitatory neurotransmitter in the central nervous system) in the trigeminal ganglia. The results demonstrated that the release of glutamate was significantly reduced in DG than that observed in NG ($p<0.05$; Two-way ANOVA, Bonferroni′s Test) (Figure 3). There is no statistical difference between DG and IG at days 0, 1, 6, and 12 after orthodontic appliance placement ($p>0.05$) (Figure 3). These results suggest that, although the diabetes potentialize the inflammatory response in the gingival tissue the activation of neuronal structures in the trigeminal tissue was reduced.

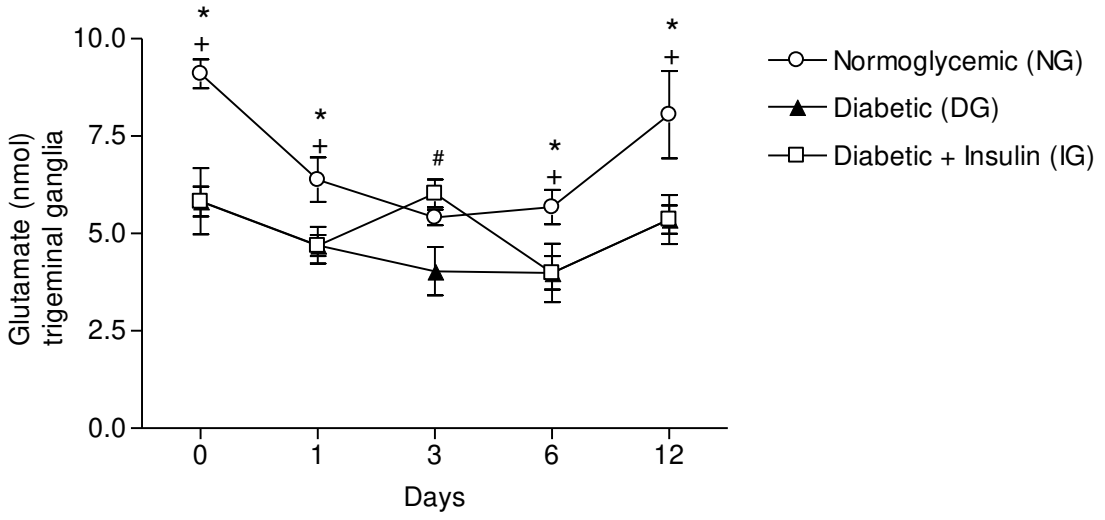

Figure 3. **Diabetes reduces the release of glutamate in trigeminal ganglia**. The release of glutamate in the trigeminal ganglia in DG is significantly lower than that observed in NG at days 0, 1, 6 and 12

($p<0.05$: Two-way ANOVA, Bonferroni's test). The release of glutamate was significantly lower in DG than that IG at day 3 ($p<0.05$). The release of glutamate was significantly lower in IG than that NG at days 0, 1, 6 and 12. The symbol (+) indicates statistical difference between DG and NG groups. The symbol (*) indicates statistical difference between NG and IG groups. The symbol (#) indicates statistical difference between DG and IG groups.

To confirm previous results it was evaluated the axon reflex of peripheral nociceptive neurons on gingival tissue measuring the release of neuropeptides substance P (SP) and the calcitonin gene-related peptide (CGRP). The results show that the release of SP in gingival tissue was significantly higher in DG than that NG after 1 day of orthodontic appliance placement ($p<0.05$; Two-way ANOVA, Bonferroni′s Test). On the other hand, at 3, 6 and 12 days after orthodontic treatment the release of SP was significantly lower in DG than that NG ($p<0.05$) (Figure 4A). The release of SP in gingival tissue was significantly lower in IG than that NG at days 1, 3 and 6. The release of SP in gingival tissue was significantly higher in DG than that IG at days 1 and 3 (Figure 4A).

The release of CGRP in gingival tissue was significantly lower in DG than that NG after 1, 3, 6 and 12 days of orthodontic appliance placement ($p<0.05$; Two-way ANOVA, Bonferroni′s Test) (Figure 4B). The release of CGRP in gingival tissue was significantly lower in DG than that IG after 1, 3 and 12 days of orthodontic appliance placement ($p<0.05$) (Figure 4B).

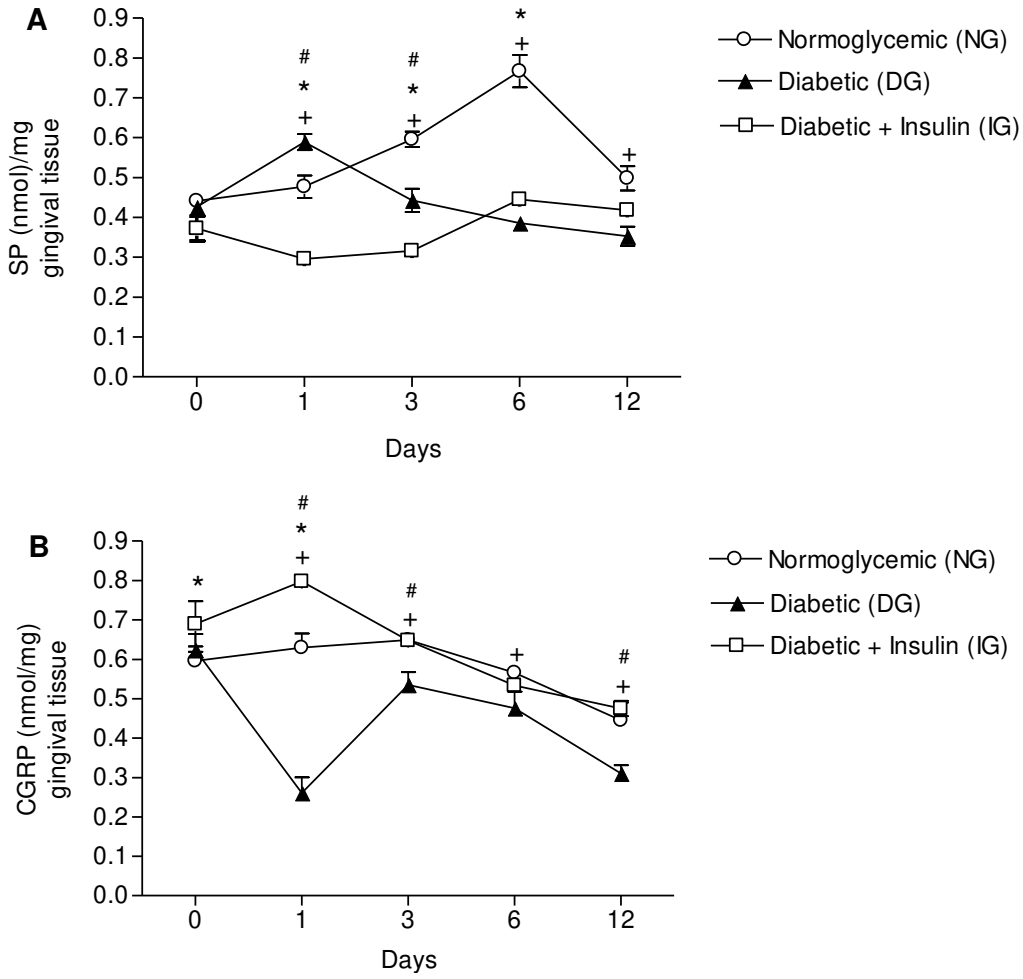

Figure 4. **Diabetes reduces the release of SP and CGRP in gingival tissue. (A)** The release of SP in gingival tissue was significantly higher in DG than that NG at day 1; and significantly lower than that NG at days 3, 6 and 12 ($p<0.05$; Two-way ANOVA, Bonferroni's test). The release of SP was significantly lower in IG than that NG at days 1, 3 and 6 ($p<0.05$). The release of SP in gingival tissues in DG was significantly higher than that IG at days 1 and 3 ($p<0.05$). **(B)** The release of CGRP in gingival tissue was significantly lower in DG than that NG at days 1, 3, 6 and 12 ($p<0.05$). The release of CGRP was significantly higher in IG than that DG at days 1, 3 and 12 ($p<0.05$). The release of CGRP was significantly higher in IG than that NG at days 0 and 1 ($p<0.05$). The symbol (+) indicates statistical difference between DG and NG groups. The symbol (*) indicates statistical difference between NG and IG groups. The symbol (#) indicates statistical difference between DG and IG groups.

Discussion

In the present study it was demonstrated that early phase of diabetes induced an increase of inflammatory response that in turn significantly increased the orthodontic tooth movement. As previously demonstrated (Braga *et al.*, 2011), the results of the present work suggest that diabetes increased the release of the inflammatory cytokines TNF-α and IL-1β.

The potentiation and persistence of inflammatory response could have several effects, such as a tendency toward greater matrix degradation or reduced capacity in repair injured tissue following bacteria-induced injury (Sodek and Overall, 1992; Liu *et al.*, 2004). Indeed, the amount of pro-inflammatory cytokines ultimately trigger the release of final mediators that act directly on the nociceptors resulting in lowering of threshold due to modulation of specific voltage dependent sodium channels (Verri *et al.,* 2006). In this way, it has been known that diabetes is associates with higher production of inflammatory cytokines that result in a maintenance and amplification of inflammation (Naguib *et al.*, 2004; Graves *et al.*, 2005), and an increased release of excitatory neurotransmitters and neuropeptides in the central nervous system, which in turn, could develop in a diabetic peripheral neuropathy (Calcutt, 2013; Osikowicz *et al.*, 2013).

Despite enhance of inflammatory response induced by diabetes as a result of orthodontic tooth movement, the results demonstrated that diabetes significantly reduced the release of glutamate in the trigeminal ganglia. These data suggest that diabetes induced a peripheral neuropathy in the trigeminal system that cause a reduction of neuronal activation.

Peripheral C-fiber nociceptors have afferent and efferent functions (Sann and Pierau, 1998). Particularly, peripheral C-fibers were responsible for the axon reflex flare reaction, which involves antidromic impulses travelling along the peripheral nerve terminal, as a result of noxious stimulus. In the injury tissue, the peripheral C-fibers release neuropeptides, such as SP, neurokinin A and CGRP (Sann and Pierau, 1998). The release of these neuropeptides in the peripheral tissues potentializes the inflammatory process and sensitization of peripheral C-fibers itself (Sann and Pierau, 1998). Thus, to confirm the effect of diabetes on neuronal activity, it was evaluated the effect of diabetes on axon reflex

of peripheral nociceptive neuron in gingival tissues. The result shows that diabetes significantly reduced the release of SP and CGRP after 3, 6 and 12 days the orthodontic appliance placement.

Although peripheral neuropathy is a common complication of diabetes, the mechanism which develops diabetic neuropathy is unclear. It is known that this sensory disturbance may be closely associated with dysfunction of nerve conduction, velocities and transmission (Borghini *et al.*, 1994; Roberts and McLean, 1997). In this way, previous studies suggest that the key pathogenetic mechanism implicated in the increased of aldose-redudase activity oxidative-nitrosative stress, protein kinase C, poly (ADP-ripose) polymerase, angiotensin converting enzyme activations, C-peptide deficiency, impaired neurotrophism and pro-inflammatory response (Obrosova, 2009). Considering that diabetes-induced animals had increased pro-inflammatory response, it is possible to suggest that the diabetes decrease the neuronal excitability in the trigeminal system which may be related with the Diacilglicerol (DAG)/ Protein kinase C (PKC) pathway.

In this way, it has been shown that hyperglycemia leads to neurovascular complications increasing the formation of DAG and consequently activation of different isoforms of PKC (Evcimen and King, 2007). PKC has been associated with vascular alterations such as increases in permeability, contractility, extracellular matrix synthesis, cell growth and apoptosis, angiogenesis, leukocyte adhesion, and cytokine activation and inhibition (Geralds and King, 2010). Considering that Na^+K^+ATPase activities are reduced in vascular and neuronal tissues of diabetic patients and animals (Winegrad, 1987; Xia *et al.*, 1995), and PKC was associated with the activation of the Na^+K^+ATPase (Evcimen and King, 2007), it is possible that early phase of diabetes in the trigeminal tissues potentialize the inflammatory response resulting in the activation of DAG/PKC pathway, inhibition of Na^+K^+ATPase and the decrease of neuronal excitability of trigeminal neurons.

Thus, considering that diabetes potentialize the inflammatory response, responsible for the orthodontic tooth movement, but on the other hand neuronal activation of trigeminal system was reduced, it is imperative the special attention of the orthodontist with orthodontic forces in diabetic people.

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CONCLUSÃO

O presente estudo sugere que o diabetes tipo 1 potencializa o processo inflamatório por meio do aumento da liberação da citocinas pró-inflamatórias TNF-α e IL1β, como resultado da movimentação dental por tracionamento ortodôntico. No entanto, apesar de acentuar o processo inflamatório, os resultados sugerem que o diabetes reduz a resposta dos neurônios periféricos do tecido gengival. Nesse contexto, foi demonstrado que o diabetes tipo 1 reduz a liberação dos neuropeptídios SP e CGRP no tecido gengival, responsáveis pelo processo denominado reflexo axônico periférico, assim como a liberação do neurotransmissor excitatório glutamato no gânglio trigeminal. Consideramos que este estudo é de relevância clínica por alertar os ortodontistas a terem uma especial atenção na aplicação de forças mecânicas para o tracionamento dental em pacientes diabéticos.

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ANEXO 1

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Manuscript JDR-14-0144 Submitted to the Journal of Dental Research

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10-Feb-2014

Dear Dr. Clemente-Napimoga:

Your manuscript, "Diabetes reduces neuronal activation during orthodontic tooth movement," has been successfully submitted online to the Journal of Dental Research.

Your manuscript ID is JDR-14-0144.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your contact information, please log in to SAGETrack at http://mc.manuscriptcentral.com/jdr and edit your user information as needed. You can also view the status of your manuscript at any time by checking your Author Center after logging into system.

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Sincerely,

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Journal of Dental Research

ANEXO 2

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O,

Certificamos que o projeto **"AVALIAÇÃO DO EFEITO DA MOVIMENTAÇÃO ORTODÔNTICA NO DESENVOLVIMENTO DE NEUROPATIA DECORRENTE DO DIABETES INDUZIDO EM RATOS"** (protocolo nº **2559-1**), sob a responsabilidade de **Profa. Dra. Maria Beatriz Duarte Gavião / Fabiana Furtado Freitas**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **07 de novembro de 2011**.

Campinas, 07 de novembro de 2011.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

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