UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

EVER ELIAS MENA LAURA

Impact of metformin on periodontal response to orthodontic forces in type 1 and 2 diabetic rats

Impacto da metformina na resposta periodontal à forças ortodônticas em ratos diabéticos tipo 1 e 2

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Tese constituída por artigos apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Biologia Oral.

Orientador: Prof. Dr. Gerson Francisco de Assis

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“O que fazemos em vida, ecoa na eternidade”

— Gladiador (2000)

ABSTRACT

Objectives: We studied the periodontal response to orthodontic forces in type 1 diabetic rats (T1D) treated with insulin and metformin and type 2 diabetic rats (T2D) treated exclusively with metformin (MET). **Materials and methods:** In article 1, T1D was induced by single injection of streptozotocin (STZ), whereas in article 2, T2D was induced by 90 days of high fat diet (HFD) with a single and low dose administration of STZ. In both articles 1 and 2 as soon as diabetes was induced in rats, an orthodontic appliance was installed to move the right upper first molar mesially for periods of 0, 3, 7, and 14 days. Samples were analyzed by micro-CT histomorphometry and immunohistochemistry for TRAP + cells. **Results:** Diabetic induction with STZ on the one hand, and HDF plus STZ on the other resulted in pathognomonic signs of T1D (hyperglycemia) and T2D (increase in body mass, insulin resistance, glucose intolerance and hyperglycemia), respectively. The addition of MET decreased blood glucose to values close to NG and better than insulin alone in T1D. In T2D, MET significantly reduced blood glucose, insulin tolerance and glucose tolerance. During orthodontic movement (OTM), T1D and T2D led to greater mesial movement, mesial inclination, mesial rotation (mesioversion), periodontal ligament spacing associated to a larger number of TRAP+ cells, and bone resorption surfaces (ORS) which were significantly reduced by MET on T2D and MET added to insulin on T1D. T2D presented maxillary osteoporosis or reduced BV / TV and BA / TA before OTM, but in T1D this occurred during OTM, however these effects were counteracted by MET. Yet, a different pattern of OTM occurs in T1D and T2D due to different bone density, and extrusion versus intrusion presented in T1D and T2D, respectively. **Conclusion:** The addition of metformin to insulin in T1D or single administration in T2D reduces the adverse effects on periodontal tissues during orthodontic movement in type 1 and 2 diabetic rats.

Keywords: Metformin; Type 1 diabetes; Type 2 diabetes; Orthodontic tooth movement; Periodontium.

RESUMO

Objetivos: Nós estudamos a resposta periodontal às forças ortodônticas em ratos diabéticos tipo 1 (T1D) tratados com metformina (MET) adicionada à insulina, e ratos diabéticos tipo 2 (T2D) tratados exclusivamente com metformina. **Materiais e métodos**: No artigo 1 a T1D foi induzida por injeção única de estreptozotocina (STZ), enquanto que no artigo 2 a T2D foi induzida por alimentação rica em gordura (HFD) por 90 dias e administração de dose única e baixa de STZ. Em ambos artigos 1 e 2 assim que a diabetes foi induzida nos ratos, um aparelho ortodóntico foi instalado para movimentar o primeiro molar superior direito mesialmente por períodos de 0, 3, 7, e 14 dias. As amostras foram analisadas por micro-CT histomorfometria e imunohistoquímica para células TRAP+. **Resultados**: A indução diabética, com STZ por um lado, e HDF mais STZ por outro, resultou em signos patognomônicos da T1D (hiperglicemia) e T2D (aumento da massa corporal, resistência insulina, intolerância à glicose e hiperglicemia), respectivamente. A adição de MET diminuiu a glicemia a valores próximos do NG e melhor do que só insulina na T1D. Na T2D a MET reduziu significantemente a glicemia, tolerância à insulina e tolerância à glicose. Durante o movimento ortodôntico (MO), T1D e T2D levaram a maior movimento mesial, inclinação mesial, rotação mesial (mesioversão), espaçamento do ligamento periodontal associado a maior numero de células TRAP+ e superfícies de reabsorção óssea (ORS) os quais foram significantemente reduzidos pela MET na T2D e a adição de MET à insulina na T1D. A T2D apresentou osteoporose maxilar ou BV/TV e BA/TA reduzido antes do MO, mas no T1D, isso ocorreu durante o MO, porem esses efeitos foram contrariados pela MET. Ainda um diferente padrão de MO ocorre na T1D e T2D pela diferente densidade óssea, e extrusão versus a intrusão apresentadas na T1D e T2D, respectivamente. **Conclusão**: A adição de metformina à insulina na T1D ou administração única na T2D reduzem os efeitos adversos no periodonto durante o movimento ortodôntico em ratos diabéticos tipo 1 e 2.

Palavras-Chave: Metformina; Diabetes tipo 1; Diabetes tipo 2; Movimento dentário ortodôntico; Periodonto.

ACRONYMS AND ABBREVIATIONS

ANOVA	Annalise of variance
BMD	Bone mineral density
BV	Bone volume
BV/TV	Bone volume / total volume
BA/TA	Bone area / total area
CEEPA	Ethics Committee FOB-USP
cm	Centimeter
DB or DBR	Disto-buccal root
EDTA	Ethylenediamine tetraacetic acid
FBGL	Fasting blood glucose levels
Fig	Figure
g	Gram
GTT	Glucose tolerance test
HA	Hyaline areas
HE	Hematoxylin – eosin
HFD	High fat diet
ITT	Insulin tolerance test
kg	kilogram
MET	Metformin
mg	Milligram
Micro-CT	Microcomputed tomography
mL	Milliliter
mm	Millimeter
mm^2	Squared millimeter
NG	Normoglycemic
OTM	Orthodontic tooth movement
ORS	Osteoclastic resorption surface
PDL	Periodontal ligament
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TRAP	Tartrate-resistant acid phosphatase

TABLE OF CONTENTS

1 INTRODUCTION

1 INTRODUCTION

DM prevalence is increasing worldwide. It reaches 37% of population in some regions (Kharroubi and Darwish, 2015). More specifically, according to de International Diabetes Federation, current global prevalence of diabetics is 425 million, they are likely to be 592 million by 2035 people, and 90% of them are Type 2 (Idf, 2019). Diabetes mellitus (DM) comprehends a certain group of metabolic diseases characterized by hyperglycemia however the most common are type 1 and 2 (T1D and T2D, respectively). T1D patients displays marked hyperglycemia. It represents a common endocrine and metabolic condition with absolute insulin deficiency especially seen in children. As consequence they display clinical polyuria, polydipsia, polyphagia, weight loss, and blurred vision (Who, 2019). T1D or insulin-dependent diabetes is also synonym of "autoimmune T1D" because T1D-associated autoantibodies produces autoimmunity causing loss of pancreatic islet β-cells in majority of the patients (Roep and Peakman, 2012), besides, mutations in more than one gene, as well as environmental factors are also related to its pathoethiology (WHO, 2019). T2D, formerly noninsulin-dependent diabetes, is more frequent in adult population and primarily related to lifestyle factors, genetics and other environmental factors (Kharroubi and Darwish, 2015). Nonetheless, T2D is increasing in children, adolescents, and younger adults due to the rising obesity, physical inactivity, and energy-dense diets (Goyal and Jialal, 2019a). Accordingly, T2D is associated to insulin-resistance, a process in which insulin secretion is uncapable to maintain euglycaemia and leads to beta cell dysfunction Additionally, obesity is linked to T2D for it contributes to insulin resistance by elevating the levels of circulating free fatty acids which in turn inhibit glucose uptake, glycogen synthesis and glycolysis. Those processes are later compensated by an increase in insulin production and secretion (Carvalheira *et al.*, 2002) which will eventually lead to pancreatic β-cells dysfunction, inappropriate glucose response and glucose intolerance (Cerf, 2013; Goyal and Jialal, 2019b).

Nonetheless, the sequence of successions and the nature of signals derived from the insulin resistant tissues to induce an appropriate beta-cell response remains unclear in the literature. (Cerf, 2013; Goyal and Jialal, 2019b). Concomitantly, adipose tissue, acts as an endocrine organ that secretes a large number of factors related to immune cell functions. Besides, adipocytes increase causes adipose tissue hypoxia and subsequent chronic inflammation (Ye *et al.*, 2007). In rodents and humans, this tissue is infiltrated by neutrophils and later by macrophages which are also positively correlated with insulin resistance (Talukdar *et al.*, 2012). Once diabetes is stablished, different detrimental effects on tissues are produced and those depend on inherent factors from both T1D and T2D.

In odontology there is well stablished bidirectional relationship between periodontitis and T1D or T2D, i.e. periodontitis itself worsening hyperglycemia and vice versa. Therefore, periodontium is brittle under diabetic condition and these detrimental effects have a significant impact in dental therapies such as orthodontics. During orthodontic tooth movement (OTM), the periodontium, a reach in cells and vascularized connective tissue, undergo an aseptic-like inflammation (Li *et al.*, 2018) in where equilibrated soft and hard tissues remodeling occurs whenever a correct amount of force is applied. Accordingly, in normoglycemic individuals, pressure site generation by mechanical stimulus promotes alveolar bone resorption due to osteoclastic activity and, in the tension site, bone matrix deposition and mineralization by osteoblastic activity (Hadjidakis and Androulakis, 2006; Krishnan and Davidovitch, 2006; Wise and King, 2008). In vitro, periodontal ligament cells undergoing compression force increase the production of factors related to osteoclast activity and survival then promoting alveolar bone resorption (Cao *et al.*, 2014; Yi *et al.*, 2016). On the tension side fibroblast experiment tension and then releases cytokines related to osteoblast activity and bone formation (Meikle, 2006). All these event change during diabetes however clinical and laboratorial studies are scarce. Regarding T1D, although a morphological

description of detrimental effects on periodontal tissues is been reported early (Holtgrave and Donath, 1989), few reports haven presented to date. Among them (Villarino *et al.*, 2011) confirmed previous reports, also (Braga *et al.*, 2011) presented a more comprehensive molecular pathway involved. However, there are orthodontic and periodontal important clinical factors to be approached such as the tooth movement pattern, periodontal width or thickness, also alveolar bone height and volume. What is more, there is almost no knowledge of what occurs ins such conditions under treatment. According to (Jonasson *et al.*, 2018), alveolar bone is unique capable of following teeth's movements; this bone formed around teeth during eruption and their PDL, thus, the longer the teeth after eruption, the larger the alveolar process. In a mature maxilla, different densities are found in the alveolar process (Jonasson *et al.*, 2018), thus, different turnover rates (Parfitt, 2013), however, how diabetes or diabetic treatment affects these factors are not fully understood.

Metformin (MET), a biguanide and an oral anti-hyperglycemic agent is the first therapy of choice in T2 diabetics. Although some side effects exist (Dujic *et al.*, 2016), it is effective because it promotes insulin sensibility, slows the release of glucose stored in the liver and reduces glucose absorption in the gut (Hostalek *et al.*, 2015). MET enhances insulin action, improves glycemic control, then leads to reduce insulin dose requirement as well as weight gain. Nonetheless, MET adjunctive therapy is not formally prescribed in T1D as in T2D is (Degeeter and Williamson, 2016), and whether to add metformin to insulin therapy in T1D is under debate . (Beysel *et al.*, 2018). In the article 1, we approached the use of MET as an adjunctive therapy of insulin in T1D rats undergoing OTM. In young Wistar rats, T1D was induced by Streptozotocin, then and orthodontic appliance was placed between the scissors and the first upper molar (M1) in order to move it mesially. Four groups that properly simulate clinical conditions related to diabetic patients were established: A control Normoglycemic (NG); diabetic (T1D); Insulin treated diabetics (I-T1D); and the proposed

MET plus Insulin treated diabetics (IM-T1D). To understand whether MET adjunction to Insulin has any effect on periodontal behavior while submitted to orthodontic forces, several analyzes were performed. Accordingly, 3D spatial position changes of the M1 or tooth movement pattern was approached by micro-CT; periodontal tissues events and remodeling were approached by micro-CT and histomorphometry to evaluate periodontal spacing, alveolar bone volume fraction and presence of hyaline tissue; finally, osteoclastic activity as the presence of positive trap cells were also recorded. All these variables may give a comprehensive panorama of the PDL behavior of T1D diabetics that are submitted to OTM, also to antidiabetic therapy such as insulin or insulin plus MET.

In regard to T2D subjected to OTM, periodontal tissues may also be frail. T2D involves other diseases such as obesity and osteoporosis, therefore, the present chronic inflammation (Talukdar *et al.*, 2012) can directly influence the periodontal response related to force loading. To date few laboratorial studies approached T2D effects on tooth movement and all of them point periodontal response to be altered during orthodontic tooth movement (Plut *et al.*, 2015; Sun *et al.*, 2017; Gomes *et al.*, 2018). Regarding treatment, (Sun *et al.*, 2017) reported MET use in T2 animals subjected to OTM by histologic means. They pointed the outcomes as evidence that MET reverses T2D deleterious effects in such conditions. Those assumptions were based on higher OTM rate recorded manually in silicone models and cellular activity recorded in histological sections. In the article 2 we present a more complete scene of the T2D and MET effects on OTM. For that purpose, T2D was induced by high fat diet (HFD), a modified form from AIN-93 standard diet, associated to streptozotocin for it resembles T2D development similar to humans (Vatandoust *et al.*, 2018); therefore, rats underwent glucose tolerant test (GTT) and insulin tolerant test (ITT) to conferee prediabetes and insulin resistance (IR) presence in these animals. As T2D was stablished, an experimental group was treated by MET daily (M-T2D) and other groups were untreated (T2D) and

normoglycemic (NG). Outcomes after 14 days of OTM were evaluated by micro-CT and histology. The impact of both T2D and MET on periodontal tissues during orthodontic tooth movement is not fully understood. Although orthodontics is commonly performed in young patients, adult population seeking for orthodontic treatment is growing and dentists have to face proportionally the adult-related systemic diseases such as T2D (Almadih *et al.*, 2018).

Orthodontic studies related to diabetes are scarce. Additionally, it seems that there exists a tendency of generalization for both T1D and T2D among orthodontic review studies (Najeeb *et al.*, 2017; Almadih *et al.*, 2018; Chauhan *et al.*, 2018). Thus, we aimed to study metformin effect on periodontal tissues during orthodontic tooth movement in T1D and T2D rats by micro-CT, histology and immunohistology for TRAP.

2 ARTICLES

2 ARTICLES

This thesis comprises two articles:

ARTICLE 1 – Metformin as an add-on to insulin improves periodontal response during orthodontic tooth movement in type 1 diabetic rats

ARTICLE 2 – Metformin therapy to prevent periodontal breakdown after orthodontic forces in type two diabetic rats. A micro-CT, Histomorphometric and immunohistochemical evaluation

2.1 ARTICLE 1 – *"This is the peer reviewed version of the following article: [***Metformin as an add-on to insulin improves periodontal response during orthodontic tooth movement in type 1 diabetic rats***], which has been published in final form at [*https://aap.onlinelibrary.wiley.com/doi/abs/10.1002/JPER.18-0140*]. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions."*

Metformin as An Add-On to Insulin Improves Periodontal Response During Orthodontic Tooth Movement in Type 1 Diabetic Rats

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Running title: Metformin plus insulin in periodontal response to OTM in T1D

One sentence summary: Metformin plus insulin therapy ameliorates glycemic control and the periodontal tissue response to orthodontic forces in T1D rats.

ABSTRACT

Background: Type 1 diabetes (T1D) is associated with delayed tissue healing and bone loss. Periodontal tissues during tooth movement (OTM) in T1D and under diabetic treatment are poorly understood. We aimed to study the effect of metformin as an add-on to insulin therapy on periodontal structures during OTM in T1D rats.

Methods: Rats were divided into normoglycemic (NG, n=20) and **s**treptozotocin-induced diabetic groups that were untreated (T1D, n=20), treated with insulin (I-T1D, n=20), or treated with insulin plus metformin (IM-T1D, n=20). After 7 days of treatment, the first right upper molar (M1) was moved mesially. At day 14, the pattern of OTM and the periodontal tissues were analyzed by micro-CT, histomorphometry and immunohistochemistry for TRAP.

Results: In T1D, major osteoclastogenic activity and bone loss versus other groups were confirmed by a greater TRAP-positive cell number and reabsorption surface on both the pressure and tension sides for 14 days ($p<0.01$). Additionally, we observed low bone volume density. Metformin plus insulin resulted in a daily insulin dose reduction and major glycemic control versus I-T1D. Although no significant differences were observed between I-T1D and IM-T1D, the tooth displacement and inclination, periodontal ligament thickness and alveolar bone density on the pressure side in IM-T1D were similar to that of NG ($p>0.05$).

Conclusion: Antidiabetic treatment reduces severe periodontal damage during applied orthodontic force in T1D untreated rats. Metformin as an add-on to insulin therapy resulted in glycemic control and a periodontal tissue response to orthodontic forces that was similar to that of normoglycemic rats.

KEYWORDS: Diabetes Mellitus Type 1, Insulin, Metformin, Orthodontic Tooth Movement, Alveolar Bone Loss

INTRODUCTION

Type 1 diabetes (T1D) is a chronic metabolic disease characterized by hyperglycemia, resulting from the absence of insulin secretion[1]. In addition to diabetic retinopathy, nephropathy and neuropathy[2], T1D has been characterized by poor bone health[1,3]. Recent studies relate T1D to a high prevalence of osteopenia and osteoporosis as a consequence of impaired bone turnover[3,4]. In the clinical dental practice, young diabetics show delayed skeletal development followed by diminutions in cephalometric parameters, predisposing them to malposition of teeth[5]. Adult diabetic patients also show occlusal problems related to periodontal degradation and tooth loss. This leads to nutritional deficiencies, psychosocial consequences and impaired quality of life[6]. In these circumstances, orthodontic treatment is indicated. Additionally, orthodontic force induces a complex adaptive tissue response and the metabolic alterations associated with T1D interfere with this process resulting in a different rate and pattern of tooth movement[7]. Regarding tooth movement and its periodontal tissue response in T1D, the literature is scarce. Some studies suggest that in T1D individuals, satisfactory orthodontic results with a proper periodontal response can be obtained if hyperglycemia is well controlled[8,9]. Therefore, if euglycemia is poorly maintained, a real risk of periodontal breakdown contraindicates orthodontic treatment[8,9].

Insulin is an anabolic agent for bone[10]. A lack of insulin leads to an impaired osteoblast function giving rise to a low bone-turnover state which results in a decreased peak bone mass during puberty[3,4] and a reduced bone mineral density during adult life[4,11,12]. However, the use of insulin therapy alone cannot restore bone quality and strength[13-15]. A cohort study using data from The Health Improvement Network (THIN) evaluated the incident fracture of 30,394 participants with T1D and 303,872 without it. The incidence of fractures was greater in T1D individuals (31.1% vs. 25.1% in males, 39.3% vs. 32% in females; $P < 0.001$)[14]. Another far reaching study from the National Health and Nutrition Examination Survey (NHANES) found that T1D diabetic subjects have lower bone mineral density (BMD) at the total femur and femoral neck, and consequently 4.7 times more risk of

osteoporosis than T2D individuals[15]. Even after intensive insulin treatment the elevated fracture risk is still observed in T1D patients [16] and animal models[13,17].

Thus, it should be taken in consideration the possible adjunctive therapies influence on bone metabolism. The insulin-sensitizing metformin showed in an *in vitro* study a positive effect on osteoblastic replication, differentiation and mineralization via AMPK activation[18]. In ovariectomized rats metformin significantly decreased the osteoclast (TRAP cells) recruitment and increased BMD by stimulating osteoprotegerin secretion and reducing RANKL expression[19]. Curiously, oral administration of metformin in combination with rosiglitazone in non-diabetic rats, prevented the adverse effects of TZDs on bone tissue[20]. In animal models for dental research, systemic administration of metformin to non-diabetic rats with apical lesions reduced osteoclasts number and inhibited alveolar bone resorption[21]. Adittionally, topical application of metformin gel in ligature-induced periodontitis decreased the inflammatory response, oxidative stress, and bone loss[22] . However, reports on clinical use of metformin are scarce and contradictory. Studies involving T1D and T2D patients have shown reduced fracture risk with biguanides/metformin therapy[23,24]. In contrast, it has been demonstrated no association between the risk of fractures and metformin therapy[25].

Currently, metformin add-on to insulin therapy is a treatment dogma that needs to be addressed[26]. Insulin alone showed some benefits on periodontal structures during OTM[27-29]**.** However, the potential protective effects of metformin plus insulin on these tissues is still unknown. We hypothesized that the periodontal response after application of orthodontic forces was more favorable in T1D individuals receiving a combined treatment of insulin plus metformin versus those that received only insulin. Thus, we explored metformin's potential effect on the pattern of tooth movement, periodontal ligament dimensions, alveolar bone changes, and the role of osteoclasts on both the pressure and tension sides of the periodontium of T1D rats treated with insulin alone and insulin plus metformin.

MATERIAL AND METHODS

Animals

A hundred 8-week-old Albinus *Wistar* male rats weighing between 300 g to 350 g were kept in plastic cages at a room temperature of 22 ± 2 °C, 55 ± 15% humidity, a 12 h light/12 h dark cycle and an exhaust system with 15 exchanges/h. **Food** and **water were given ad libitum**. This study was conducted with the approval of the Ethics Committee of Bauru School of Dentistry – Sao Paulo University (CEEPA Nº 33/2013).

Experimental design and groups (Figure 1A$_1$)

T1D was induced in 80 rats by a single intraperitoneal injection of 47 mg/kg of streptozotocin[‡] with a success rate of 78.75% (17 rats died), the remaining 20 animals received saline solution (NG). Seven days after induction, the fasting blood glucose level (FBGL) was measured from the tail tip using a digital **glucometer**[§]. Only animals with a FBGL greater than 180 mg/dl and clinical signs of T1D (n=60) were considered (three rats were excluded from the study). Animals were randomly divided into 4 groups: NG (n=20) and T1D (n=20) as the control groups treated with saline solution, I-T1D (n=20) treated with a sub-therapeutic dose of 2 IU of slow-release insulin[ǁ] in the morning and 2 IU of regular insulin[¶] plus 2 IU of slow-release insulin[ǁ] in the evening summing a total daily insulin dose of 6UI[30], and IM-T1D (n=20) treated with insulin as in I-T1D plus 150 mg/kg of metformin given by gavage daily until the end of each period. During treatment, physical state and animal behavior were monitored at morning and evening daily, and FBGL every three days. Insulin adjustment was realized in IM-T1D to avoid hypoglycemic episodes (FBGL <70mg/dL). Clinical and laboratory assessments were performed on the induction day, seven days after induction/initial diabetes treatment and at the end of each trial period: 0 (baseline), 3, 7 and 14 days (n=5/group) (Figure 1A).

Application of orthodontic force (Figure 1A$_2$)

OTM was performed by a trained researcher following a standardized protocol. Animals were anaesthetized with an intraperitoneal injection of 80 mg/kg ketamine chloridrate[#] and 8 mg/kg xilazine chloridrate[#]. A 9-mm nickel-titanium, 0.010 in × 0.030 in closed coil spring[**] was used (Figure 1A$_2$). Then, a 0.008 inch stainless steel (SS) ligature[††] was threaded through the contact between the first (M1) and second (M2) right maxillary molars and attached to the spring. A second 0.008 inch SS ligature was placed around the incisors, and the spring was activated to produce a continuous force of 50 g.

Maxillae collection and microcomputed tomography analysis (Figure 1B)

The animals were sacrificed by anesthetic overdose. Then, the maxilla was removed and fixed in 10% phosphate buffered formalin for 24h. Maxillae were scanned using a micro-CT[‡‡]. The X-ray tube voltage was 80 kV, and the current was 300 μA, with a 0.5 mm thick Cu+Al filter. Exposure time was 530 ms at 0.5° intervals with a scanning angular rotation of 180°. The projections were reconstructed using 3D-reconstruction software[§§]. A geometric alignment of M1 was performed using the 3D registration function software[‖] in the transaxial plane at the palatal plane level, the coronal plane at the level of the center of distobuccal root, and the sagittal plane at the level of the distobuccal root and parallel to the palatal suture (Figure 1B$_1$). The tooth movement pattern was measured using landmark points on the M1 crown and roots as described by Xu et al., (2013)[31] using 2D/3D image analysis software[¶¶]. The same investigator performed all measurements three times, with 15 days between each assessment using the mean value as the final measurement. For reproducibility, measurements were performed 10 times in one maxilla and the standard deviation was 2%. Linear measurements (2D) in the sagittal view (Figures 1B$_{1-5}$) were: a) mesial movement as the distance between the most convex point of the maxillary M1 and M2 (Figure 1B$_2$); b) vertical movement as the distance from the bottom of the first molar's distal sulcus to the palatal plane (Figure 1B$_3$); and c) periodontal ligament (PDL) thickness on the pressure and tension sides as the distance between the

edge of the alveolar bone and the distal (tension side) and mesial (pressure side) surface of the distal-buccal root (Figures $1B_{4-5}$). Angular measurements (Figures $1B_{6-8}$) were: a) mesial inclination angle by the intersection of the mesial root axis line and the palatal plane in the sagittal view (Figure $1B_6$); b) palatal inclination angle by the intersection of the distopalatal root axis line and the palatal plane in the coronal view (Figure $1B_7$); and c) axial rotation angle by the intersection of the palatal suture and a line running through the midpoints of the mesial and distobuccal roots in the axial view (Figure $1B_8$). Inter-radicular bone volume density or BV/TV (Figure $1B_9$) was performed following a 3D region of interest (ROI) starting coronally by a line drawn among the roots in the M1 furcation, continuing in the apical direction in 150 consecutive scan slices (1.5 mm coronal to apical from the furcation).

Histomorphometric and immunohistochemical analysis (Figure 1C)

Laboratorial procedures

The maxillae were decalcified in 4.13% EDTA[##], pH 7.2 at room temperature for 8 weeks. Hemimaxillae were subjected to histological processing. Semi-serial 4μm thick sagittal sections showing the coronal and radicular portion of the mesial and distobuccal root of the upper M1 were obtained. Three sections were submitted to immunohistochemistry using an indirect immunoperoxidase method for tartrate-resistant acid phosphatase[***] as a marker of osteoclasts.

Bone histomorphometry and TRAP positive cell number determination (Figure 1C)

A single observer (EML) analyzed the slides. Periodontal tissue images of the tension and pressure sides of the distobuccal root (Figure $1C_1$) were obtained using a high-resolution digital camera[†††] attached to a microscope[‡‡‡] and a computer with image capture software [§§§]. On both sides, a total area of 900,000 μm^2 was evaluated (Figure $1C_1$). The number TRAP-positive osteoclasts per mm2 of tissue (NcTRAP+) and the percentage of osteoclastic resorption surface (ORS) were calculated by the relationship between bone surface in contact with osteoclasts by the total surface

(Figure $1C_2$). In addition, the bone area density (BA/TA) and hyalinized area density were obtained as shown in Figure $1C_3$.

Statistical analysis

The total sample size was estimated as being 80 (5 in each group) using a software[III]. Settings were 0.05 alpha level, 0.80 power, effect size of 0.40 (large) and degree of freedom of 3. The normality of the distribution of each variable was assessed by means of the Kolmogorov-Smirnov test, then Levene's test was used to verify the homogeneity of variances. The data from clinical observation (glycaemia, water and food intake), mesial movement, ORS, hyalinized areas and NcTRAP+ did not pass homogeneity of variance thus they were assessed by a non-parametric Kruskal-Wallis test followed by **Tukey's** HSD test. Other micro-CT measurements and BA/TA showed normal distribution and homogeneity of variances thus they were submitted to two-way ANOVA with interaction effect (groups and experimental periods) followed by **Tukey's** HSD test. A $p<0.05$ significance level was set for all statistical analyzes in the software[¶¶¶].

RESULTS

Clinical laboratory data (Table 1)

On the diabetes induction day (-14 days), body mass, blood glucose level and daily water and food intake were similar in all groups. Seven days after induction or at the starting day of diabetic treatment (-7 day), parameters remained stable in the NG group. In the diabetic groups, the body weights were reduced by 19.1% while the blood glucose level and daily water and food intake increased by 421.6%, 231.0% and 55.0%, respectively ($p<0.001$), showing the severity of the disease. After 7 days of anti-diabetic treatment (day 0) in I-T1D and IM-T1D, the glycemic level reduced by 55.0% ($p<0.0001$), while the water intake and food intake were reduced by 40.0% ($p=0.0005$) and 29.0% ($p=0.0006$), respectively, showing a favorable response to both therapies. During the force application, a reduction in daily water and food intake at 3 days was observed in all groups and was

subsequently increased up to 14 days, suggesting an adaptation period after device installation. At 14 days, the glycemic value in I-T1D was 47% ($p<0.001$) higher than NG although it was similar to IM-T1D.

Micro-CT analysis

M1 displacement and tipping in response to force

In the loaded M1 from day 0 to day 14, the amount of tooth movement gradually increased for all groups ($p<0.001$), see Figures 2A and 2B. However, at 14-days, T1D and I-T1D displayed greater mesial displacement (mean of 226.4µm) while NG and IM-T1D (mean of 136.5µm) did not show statistically meaningful differences from 3 to 7 days. At 14-days, a statistically significant M1 extrusion (Figures 2A and 2C) and mesial tipping (Figures 2A and 2D) were observed in T1D (232µm and 13°, respectively) and I-T1D (134µm and 6.3°, respectively) while in NG, I-T1D and IM-T1D the vertical position and mesial inclination angle were maintained during all periods. No palatine inclination (Figure 2E) or axial rotation (Figures 3A and 3C) were observed in loaded M1 of T1D and I-T1D rats, although a slight mesial rotation in NG (2.4°) and IM-T1D (3.52°) was observed.

Periodontal thickness and alveolar bone density

In T1D, an unfavorable periodontal response to mechanical loading on both sides favored alveolar bone loss (see Figure 3). At 3 days, the values of the periodontal thickness on the pressure side (Figures 3B and 3D) reduced in NG and IM-T1D (mean of 108.7µm at 0 day for 46.2 at 3 days; $p<0.0005$), while in T1D and I-T1D only tendency towards reduction was observed (mean of 113.3µm at 0 day for 70.5 at 3 days). On the tension side (Figures 3B and 3E), the PDL thickness increased in all groups (mean of 116.9µm at 0 days versus 202.0µm at 3 days). Between 3 and 14 days, the PDL thickness of the pressure and tension sides in NG, I-T1D and IM-T1D were reduced close to that of 0 days (mean of 104.2µm on the pressure side and 167.6 µm on the tension side, respectively; $p<0.001$).

However, in T1D, a high increase of PDL thickness was observed on both the pressure (191µm) and tension sides (278.9µm) at 14 days.

The interradicular BV/TV (Figures 3A, 3B, and 3F) was similar among groups at 0 days (mean of 82.8%). This value remained stable in NG (82.77%). However, a significant reduction was observed in T1D rats until 14 days (BV/TV=58.27%; $p<0.001$), while in I-T1D (BV/TV=67.45%) and IM-T1D (BV/TV=75.46%), a slight decrease occurred but insignificant. Observation of the 2D-microCT axial images (Figure 3A) revealed greater interradicular bone loss in T1D followed by I-T1D when compared to IM-T1D and NG.

Immunolabeling pattern for TRAP and morphometric changes of periodontal tissues during tooth movement

The major osteoclastogenic activity and bone loss was seen in T1D by TRAP cell immunolabeling on both the pressure and tension sides of the distobuccal root (Figures 4A and 5A) and the morphometric NcTRAP+ (Figures $4B_1$ and $5B_1$), ORS (Figures $4B_2$ and $5B_2$) and BA/TA (Figures $4B_3$ and $5B_4$) results.

On the pressure side (Figure 4) at day 0, few NcTRAP+ cells (6 cells/mm^2), a small ORS (3.8%) and BA/TA (47.5%) were seen in all groups. At 3 days, a reduction in PDL thickness, the presence of a hyalinized area and a peak in NcTRAP+ cells were observed in all groups. However, although ORS did not show significant differences among the groups (mean of 25.8%), the NcTRAP+ cell number was greater in T1D (163 cells/mm^2) than other groups (mean of 108 cells/mm^2) at 3 days. This increase in osteoclastic activity was accompanied by a decrease in BA/TA at 7 days in all groups (mean of 35.1%; $p<0.001$). Between 7 and 14 days, the NcTRAP+ cells and ROS decreased in all groups (mean of 64 cells/mm^2 and 15%, respectively). Nonetheless, a significant BA/TA reduction was still seen in T1D (21.3%; $p<0.0001$). It was followed by I-T1D (30.8%; $p<0.002$), while in IM-T1D and NG it returned to values similar to day 0 (BA/TA mean of 43%).

On the tension side (Figure 5) at day 0, we observed an NcTRAP+ cell number of 14 cells/mm^2, an ORS of 5.6% and BA/TA of 48% in all groups. At day 3 in all groups, an increase in periodontal thickness, NcTRAP+ cells/mm^2 (72 cells/mm^2; $p<0.02$) and ORS (33.8%; $p<0.03$) were observed, but BA/TA was decreased to 31.7% $p<0.03$. Between 3 and 14 days in NG, I-T1D and IM-T1D showed a tendency of reduction of NcTRAP+ cells and ORS accompanied by new bone formation and reestablishment of periodontal thickness and BA/TA (mean of 42.7%). In T1D, the NcTRAP+ and ORS remained constant until 14 days (85 cells/mm^2 and 37.6% at 14 days, respectively) but BA/TA lowered (23% at day 14; $p<0.05$).

DISCUSSION

This study shows altered periodontal tissue recovery in response to orthodontic forces in hyperglycemic rats. In addition, we found that insulin alone and insulin plus metformin substantially preserved periodontal tissues and controlled the osteoclastic activity and tooth movement pattern similar to non-diabetic rats. However, in the overall therapy performance, metformin as an add-on to insulin was advantageous over insulin alone due to better glycemic control and the effects on periodontal tissues.

Primarily, both treatments achieved satisfactory glycemic control. Nevertheless, the insulin treatment alone (I-T1D) could not achieve euglycemia, which is in agreement with previous reports of STZ[32] and alloxan[33] T1D models. Small insulin dose variations in rats may lead to hypoglycemia due to individual variability and the size of these animals. Similarly, in juvenile T1D, even slight hyperglycemia cannot be avoided because a higher insulin dose may also lead to hypoglycemic episodes[34]. The metformin adjunctive therapy with insulin resulted in a reduction of daily insulin requirements after 10 days of treatment (3-days of OTM) and improved glycemic levels closer to those of NG. Dosage of this combined therapy in rats has not yet been reported. In T1D people, metformin is quite frequently used off-label for safe insulin-sparing and HbA1c reductions[35,36]. In obese T1D patients, insulin plus metformin also decreased insulin requirements and increased whole-body glucose uptake[37]. However, in a 10 year retrospective study, metformin plus insulin did not show more significant effects regarding insulin sparing[38].

Tooth displacement relies on the force magnitude, direction, teeth, and the patient [39]. In the normoglycemic group, the three OTM phases[40,41] were produced with no dangerous effects on the periodontium. Thus, the force used was not heavy or excessive. The initial tooth displacement at 3 days was accompanied by a high reduction of PDL thickness on the pressure side and conversely an increase on the tension side. An increasing M1 mesial displacement observed until day 7 was followed by a post-lag such as phase with a linear movement pattern until 14 days. No significant changes in tipping or vertical position and only a slight axial rotation in the force direction was noticed. Regarding bone volume, previous micro-CT studies suggested that BV/TV was reduced in NG during tooth movement[42,43]. In the present work, the interradicular BV/TV remained stable during the study.

As there are few studies on tooth movement in T1D[27,28], our study shows that 50g force in T1D individuals promote greater mesial movement when compared to NG. Additionally, we observed mesial tipping, vertical extrusion and reduced mesial axial rotation. It was also accompanied by a greater reduction of interradicular BV/TV and a greater PDL thickness than NG at day 14 on both sides. In contrast, a lower rate of tooth displacement[28,44] and alveolar bone turnover[45] in T1D rats without treatment was reported. These studies do not agree with the substantial evidence linking T1D to osteopenia[3], osteoporosis[12] and related cellular and molecular mechanisms[3,34], or with studies showing a higher rate of tooth displacement and root resorption in T1D individuals[46,47]. Therefore, our results suggest that T1D induces fragile bone that cannot support light forces which in turn become too excessive for the periodontal structures leading to breakdown and a different OTM pattern. Generally, the impaired bone metabolism in T1D children and adolescents reduces peak bone accrual, predisposing the adult to osteopenia and osteoporosis[3]. T1D insulin treated animals have shown a similar rate of tooth movement as NG[27,28], but in our work, a different OTM pattern was observed. The I-T1D group showed an OTM rate and vertical displacement similar to T1D but with major preservation of periodontal tissues. Insulin usage in type one diabetics is mandatory, however, they still show moderately low bone mineral density,[11,12] which may lead to an increased amount of tooth movement. This is explained by the slight hyperglycemia in insulin treated T1D individuals that affects bone structure, strength and quality[34]. Nevertheless, in IM-T1D, a tendency of OTM pattern

and bone volume fraction more proximate to NG was observed. Previous works showed that metformin counters the harmful effects of diabetes on bone[48] and attenuates some anti-inflammatory and autoimmune reactions,[49] so better performance compared to insulin alone could be anticipated.

Periodontal tissue remodeling depends on forces that have to go beyond the threshold that overcomes the intrinsic resistance of the PDL[39]. This will produce cellular responses in the PDL and bone leading to tissue remodeling[41]. In T1D animals, earlier reports[29,50] described reduced bone recovery, weak PDL and microangiopathies as responses to orthodontic forces. More comprehensively, in our work, these changes were accompanied by intense osteoclastic activity, a high percentage of ORS, large hyalinized areas and cell death leading to continuous loss of periodontal structures and decreasing the alveolar BA/TA until 14 days. This is explained by an imbalance between bone formation and resorption[29] and the formation of advanced glycation end-products that also increase osteoclastogenesis in T1D[1].

Although the insulin and insulin plus metformin treatments showed similar NcTRAP+ cells and ORS versus NG at 14 days on both sides, the BA/TA of IM-T1D in the pressure was better preserved compared to I-T1D. Furthermore, fewer hyaline areas compared to T1D and a similarity to NG were also evident on the pressure side which did not occur in I-T1D. Osteoclastic activity and bone resorption during OTM on the tension side have been rarely described in NG or T1D individuals. After treatment between 3 to 14 days, the NcTRAP+ cells and ORS were reduced nearly to baseline (day 0) and were accompanied by an increase in bone formation on both the pressure and tension sides. In some studies, insulin alone normalized osteoclast number[27-29]. Nevertheless, the addition of metformin showed better glycemic control and a slight tendency for better healing. A direct osteogenic effect of metformin[18] on increased BMD and decreased TRAP+ cells was previously described in ovariectomized rats[19].

Hyperglycemia undoubtedly contributes to periodontal fragility, and whether to add metformin to insulin therapy is still an important dilemma that needs to be addressed. In this study, we provide evidence that metformin addition to insulin has an advantage over insulin alone during OTM

and periodontal turnover via direct osteoclast resorption avoidance and bone formation stimulus. However, other contributing mechanisms still require further study.

CONCLUSIONS

In normoglycemic rats, the force applied in M1 produces an expected tooth movement with insignificant hyalinization and controlled PDL and alveolar bone remodeling. In untreated T1D animals, the force applied exceeds the adaptive capacity of the affected tissues. In addition, this causes cell death, extensive tissue hyalinization and alveolar bone loss leading to a different tooth movement pattern. Metformin as an add-on to insulin, besides an insulin sparing effect, controls glycemia closer to NG values. Consequently, this combination avoids different tooth movement patterns and ameliorates the periodontal response, thus reducing tissue damage and increasing the recovery better than insulin alone and similar to NG.

FOOTNOTES

‡ Sigma Chemical Co., St. Louis, MO, USA

§ Accu-Chek, Roche Diagnóstica, São Paulo, SP, Brasil

‖ Glargine Lantus, Sanofi-Aventis, Paris, France

¶ Humulin R, Lilly, Indianapolis, Indiana, USA

Ceva Saúde Animal Ltda., Paulinia, SP, Brazil

** Rocky Mountain Orthodontics. Denver, CO, USA

†† Morelli®, Sorocaba, SP, Brazil

‡‡ Skyscan 1176, Bruker Skyscan, Aartselaar, Antwerp, Belgium

§§ NRECON, Bruker Skyscan, Aartselaar, Antwerp, Belgium

‖‖ DataViewer, Bruker Skyscan, Aartselaar, Antwerp, Belgium

¶¶ CTAn, Bruker Skyscan, Aartselaar, Antwerp, Belgium

Titriplex III, Merk, Sao Paulo, SP, Brasil

*** 1:100 goat anti-TRAP, SC-30832, Santa Cruz Biotechnology, Dallas, Texas, US

††† Axiocam Hrc, Zeiss, Oberkochen, Jena, Germany

‡‡‡ Axioscope 2, Zeiss, Oberkochen, Jena, Germany

§§§ Axiovision 4.8, Zeiss, Oberkochen, Jena, Germany

‖‖‖ G-Power 3.1, Heinrich Heine University, Dusseldorf, Germany

¶¶¶Statistic 10.0, StatSoft Inc., Tulsa, OK, USA

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FIGURE LEGEND

Figure 1: Methodology. A) Experimental design: A1) Diabetes induction by STZ occurred 2 weeks before tooth movement (day-14), insulin or insulin and metformin treatment started at day -7 and were continued daily until the end of each period (P). Tooth movement was conducted for 2 weeks; A2) Orthodontic appliance. B) Micro-CT analysis: B1-B5) Linear measurements show the analyzed area (B1), the reference points, including mesial movement (B2), vertical changes (B3), PDL thickness on the tension side (B4) and the pressure side (B5); B6-B8) Angular measurements show the mesial inclination angle (B6) and the palatal inclination angle (B7) as measured in relation to the palatal plane; the axial rotation angle (B8) in relation to the medial palatal suture and B9) the BV/TV determination shows the manually delimited region of interest (ROI) in 150 consecutive slices (d). C) Histomorphometric analysis: C1) DB root of M1 immunostained for TRAP shows the area evaluated on the tension side (TS) and the pressure side (PS); C2) The number of osteoclasts was determined per square millimeter (NcTRAP+/mm2) and the percentage of osteoclast resorption surface (ORS) was calculated by the relation of bone surface in contact with osteoclasts (total **length of** yellow lines L1+L2..+L5) per unit b**one surface length** perimeter (blue line) X 100; C3) Bone (red) and hyalinized (yellow) area densities were manually surrounded and determined by the total area (blue dotted line area).

Figure 2: Three-dimensional micro-CT images (A) and graphics of linear (B and C) and angular measurements (D and E) of moved M1. T1D and I-T1D show major mesial movement versus NG at 14 days. Note the major distance between M1 and M2 (A), while in IM-T1D this distance is similar to NG. T1D and I-T1D show higher extrusion above the occlusal level (blue dashed line in A) and higher mesial tipping (red angle between the mesial root axis and the green dashed palatal line in A). Any change in palatine inclination is among groups (E). A reduction in periodontal bone level (yellow dashed line) in T1D and I-T1D can also be observed. In the graphics, mesial movement by Kruskal-

Wallis and Tukey tests, and other measurements by two-way ANOVA and Tukey test. Bars represent DPM and different letters indicate $p<0.05$.

Figure 3: Two-dimensional Micro-CT views (A and B) and graphics of axial rotation (C), PDL thicknesses (D and E) and interradicular BV/TV (F) of moved M1. NG and IM-T1D show mesial rotation (red angle in A) at day 14 while T1D and I-T1D are similar during all periods (blue angle in A). PDL thickness of the disto-buccal root DB (D and E) is similar in all groups at day 0 (blue arrow in B) but increased on the tension side (yellow arrow) and decreased on the pressure side (red arrow) at day 3. At day 14, it increased on both sides in T1D but returned to the initial values observed at day 0 in NG, I-T1D and IM-T1D. A tendency of increased thickness on the pressure side in I-T1D can be observed. There was a greater reduction of BV/TV in T1D than in NG, while in I-T1D and IM-T1D, the BV/TV is close to NG (see the inter-radicular alveolar bone in A and B). In the graphics, bars represent DPM and different letters indicate $p<0.05$ by two-way ANOVA followed by Tukey's test (groups and periods).

Figure 4: Photomicrography of the immunolabeling for TRAP (A) and graphics for NcTRAP+ (B1), ORS (B2) and BA/TA (B3) and volume density of hyalinized area (B4) on the pressure side. At day 0, the PDL space and bone tissue area (AB) were similar among groups. Note the absence or rare appearance of TRAP positive cells in A. At day 3, a reduction in PDL space with the presence of more extended hyalinized areas (black arrow head) in T1D and I-T1D versus NG and IM-T1D. A major quantity of NcTRAP+ cells (red arrow) are present in T1D versus NG, I-T1D and IM-T1D without ORS alteration. At day 7, a BA/TA reduction is evident in all groups. At day 14, a reduction of BA/TA is observed only in T1D and I-T1D, but their NcTRAP+ cell number and ORS are similar to NG and IM-T1D. In (A), the figures were obtained using a 40X objective, the scale bar value is 100 μm and D = dentin. In the graphics, BA/TA by two-way ANOVA and Tukey's tests, and other measurements by Kruskal-Wallis and Tukey tests. Bars represent DPM and different letters indicate $p<0.05$

Figure 5: Photomicrography of the immunolabeling for TRAP (A) and graphics of NcTRAP+ (B1), ORS (B2), and BA/TA (B3) on the tension side (B). At day 0, the PDL thickness and alveolar bone area (AB) are similar among groups. Note the absence or rare

appearance of TRAP positive cells (red arrow) in A. At day 3, an increase in NcTRAP+ cells (red arrows in A), ORS and PDL thickness associated with reduced BA/TA are observed in all groups and are more obvious in T1D. 7^{th} day outcomes appear transitional between periods 3 and 14. At day 14, these events are still obvious in T1D with a greater BA/TA reduction and increased PDL thickness. In NG, I-T1D and IM-T1D, an absence of TRAP cells and new bone formation (green arrow) can be observed. In (A), the figures were obtained using a 40X objective, the scale bar value is 100 µm and D = dentin. In the graphics, bars represent DPM and different letters indicate $p<0.05$ by Kruskal-Wallis and Tukey tests.

Table 1: Clinical data (mean ± SD of 5 samples) of fasting blood sugar (A), body mass (B), daily water intake (C) and daily food intake (D) before T1D induction (-14 days), at the start of diabetes treatment (-7 days) and all experimental periods during tooth movement (0, 3, 7 and 14 days). Different letters indicate $p<0.05$ by Kruskal-Wallis followed by Tukey's test.

Parameter	Group	Experimental period (days)					
		T1D Induction	T1D treatment	Tooth Movement			
		-14	-7	0	3	7	14
(A) Fasting blood sugar (mg/dL)	NG	80 ± 10 ^{A}	62 ± 14 ^{A}	79 ± 10 ^{A}	86 ± 9 ^{A}	93 ± 6 ^{A}	90 ± 12 ^{A}
	T1D	77 ± 10 ^{A}	326 ± 80 ^{B}	350 ± 58 ^{B}	384 ±116 ^{B}	413 ± 38 ^{B}	450 ± 105 ^{B}
	I-T1D	80 ± 9 ^{A}	358 ± 55 ^{B}	174 ± 21 ^{C}	156 ± 24 ^{C}	140 ± 21 ^{C}	133 ± 8 ^{C}
	IM-T1D	84 ± 9 ^{A}	342 ± 84 ^{B}	142 ± 26 $^{A, C}$	124 ± 27 $^{A, C}$	119 ± 18 $^{A, C}$	102 ± 7 $^{A, C}$
(B) Body mass (g)	NG	311 ± 15 ^{A}	330 ± 14 ^{A}	365 ± 11 ^{B}	343 ± 18 $^{A, B}$	352 ± 21 $^{A, B}$	355 ±19 $^{A, B}$
	T1D	315 ± 6 ^{A}	272 ± 12 ^{C}	238 ± 11 ^{D}	214 ± 15 ^{D}	213 ± 9 ^{D}	209 ± 16 ^{D}
	I-T1D	302 ± 17 ^{A}	243 ± 10 ^{C}	248 ± 7 $^{C, D}$	250 ± 17 ^{C}	268 ± 5 ^{C}	273 ± 15 ^{C}
	IM-T1D	307 ± 16 ^{A}	251 ± 16 ^{C}	266 ± 12 $^{C, D}$	272 ± 13 ^{C}	275 ± 10 ^{C}	274 ± 12 ^{C}
(C) Daily water intake (mL/rat)	NG	33 ± 1 ^{A}	34 ± 4 ^{A}	28 ± 3 ^{A}	25 ± 2 ^{A}	25 ± 2 ^{A}	27 ± 4 ^{A}
	T1D	33 ± 5 ^{A}	114 ± 9 ^{B}	149 ± 16 ^{C}	92 ± 9 ^{C}	110 ± 10 ^{C}	112 ± 15 ^{C}
	I-T1D	34 ± 3 ^{A}	105 ± 8 ^{B}	55 ± 7 ^{D}	48 ± 5 ^{D}	70 ± 5 ^{B}	68 ± 8 ^{B}
	IM-T1D	33 ± 2 ^{A}	110 ± 15 ^{B}	40 ± 5 $^{A, D}$	33 ± 3 $^{A, D}$	46 ± 5 ^{D}	47 ± 6 ^{D}
(D) Daily food intake (g/rat)	NG	23 ± 6 ^{A}	23 ± 2 ^{A}	21 ± 2 ^{A}	19 ± 7 ^{A}	22 ± 5 ^{A}	23 ± 5 ^{A}
	T1D	25 ± 2 ^{A}	40 ± 1 ^{B}	42 ± 1 ^{B}	28 ± 3 ^{A}	29 ± 2 $^{A, B}$	35 ± 4 ^{C}
	I-T1D	23 ± 1 ^{A}	36 ± 2 ^{B}	35 ± 2 ^{B}	27 ± 3 ^{A}	26 ± 4 ^{A}	26 ± 5 ^{A}
	IM-T1D	23 ± 2 ^{A}	39 ± 2 ^{B}	30 ±1,7 ^{A}	18 ± 5 ^{A}	21 ± 5 ^{A}	22 ±7 ^{A}

FIGURES

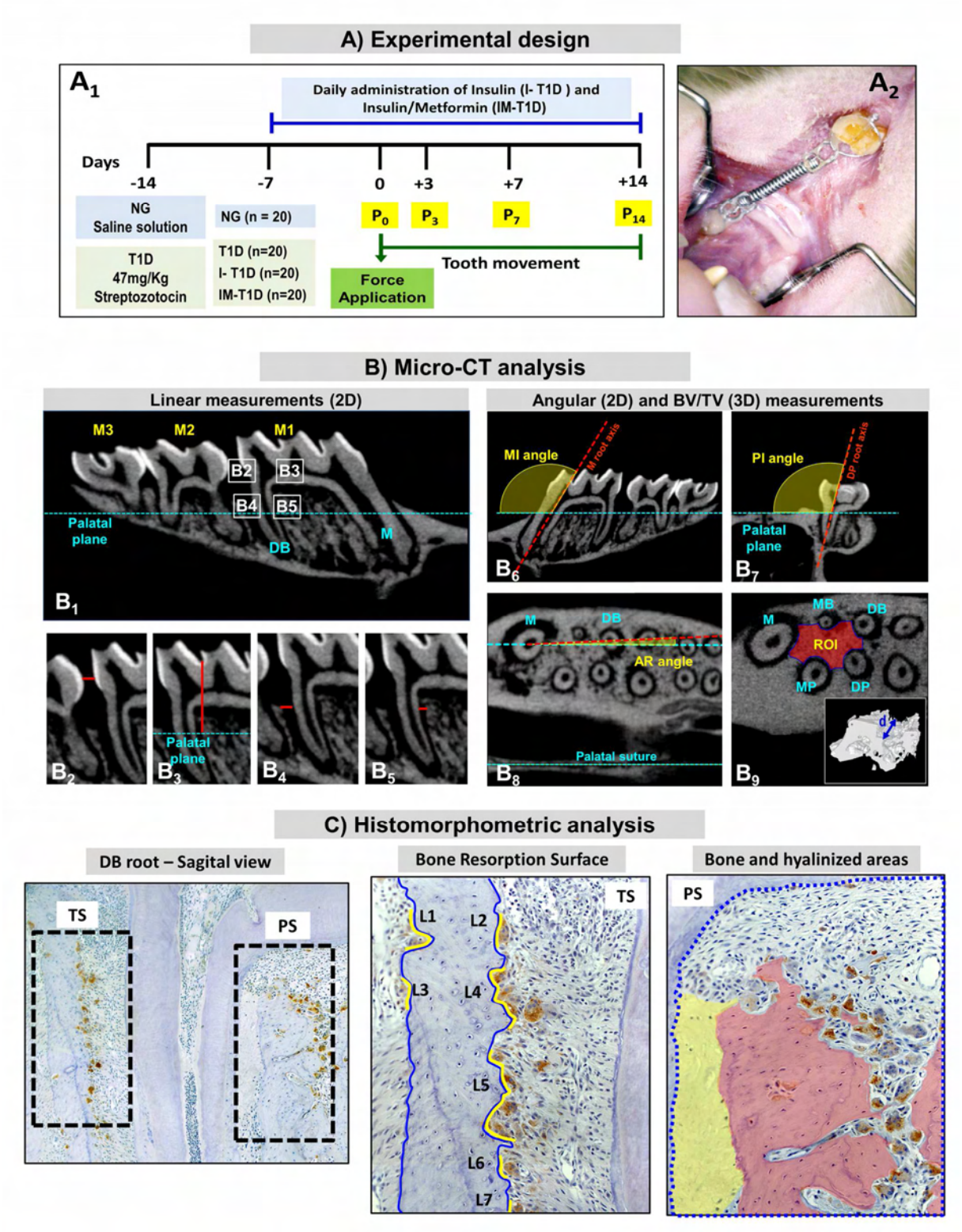
A) Experimental design
A1
Daily administration of Insulin (I- T1D) and Insulin/Metformin (IM-T1D)
Days
-14
-7
0
+3
+7
+14
NG Saline solution
NG (n = 20)
P0
P3
P7
P14
T1D 47mg/Kg Streptozotocin
T1D (n=20)
I- T1D (n=20)
IM-T1D (n=20)
Force Application
Tooth movement
A2
B) Micro-CT analysis
Linear measurements (2D)
Angular (2D) and BV/TV (3D) measurements
M3
M2
M1
B2
B3
B4
B5
Palatal plane
DB
M
B1
MI angle
M root axis
PI angle
DP root axis
Palatal plane
B6
B7
B2
Palatal plane
B3
B4
B5
M
DB
AR angle
Palatal suture
B8
MB
DB
M
ROI
MP
DP
B9
C) Histomorphometric analysis
DB root – Sagital view
Bone Resorption Surface
Bone and hyalinized areas
TS
PS
TS
L1
L2
L3
L4
L5
L6
L7
PS

Figure 1

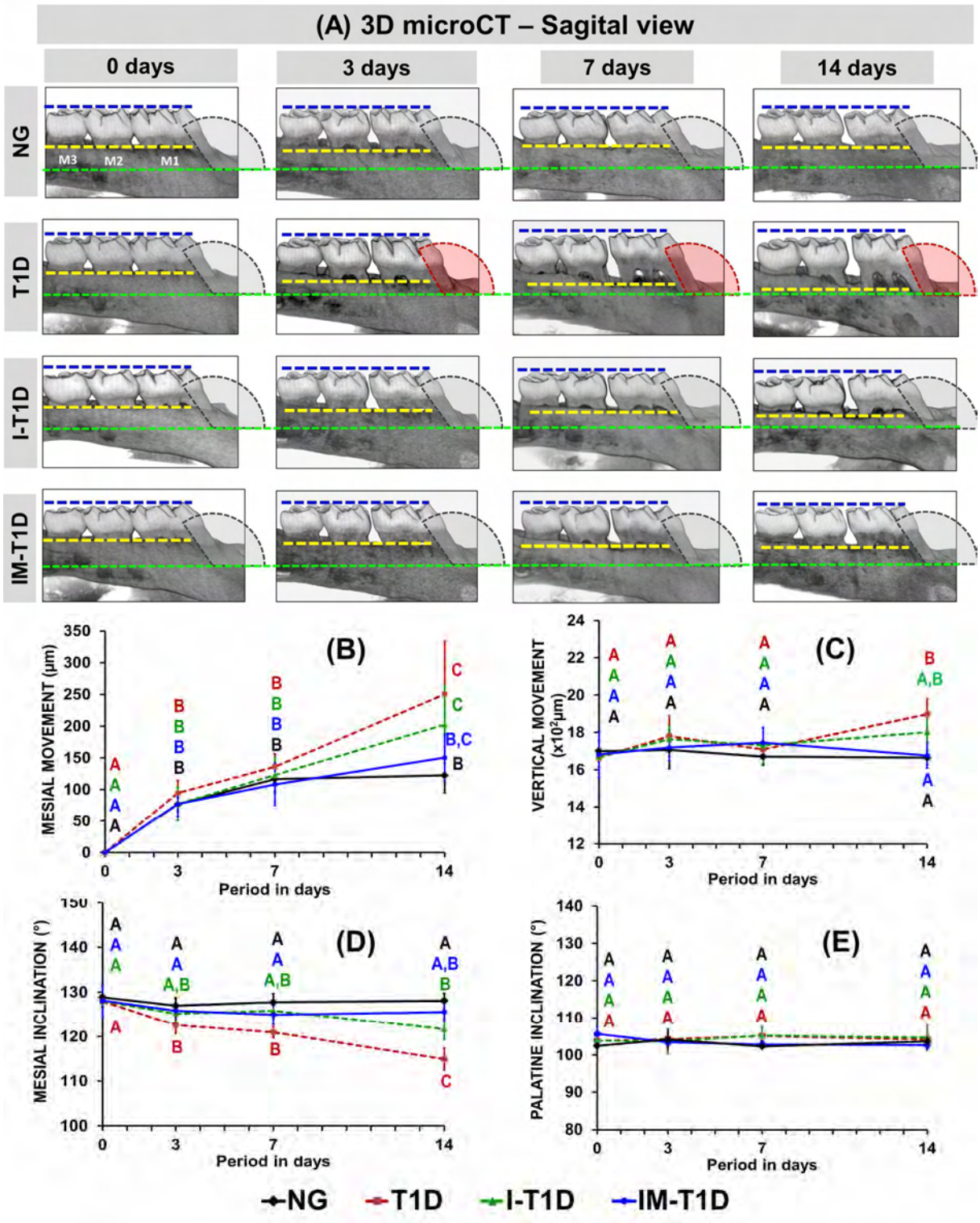
(A) 3D microCT – Sagital view
0 days
3 days
7 days
14 days
NG
T1D
I-T1D
IM-T1D
M3
M2
M1
(B)
MESIAL MOVEMENT (µm)
Period in days
(C)
VERTICAL MOVEMENT (x10³µm)
Period in days
(D)
MESIAL INCLINATION (°)
Period in days
(E)
PALATINE INCLINATION (°)
Period in days
NG
T1D
I-T1D
IM-T1D

Figure 2

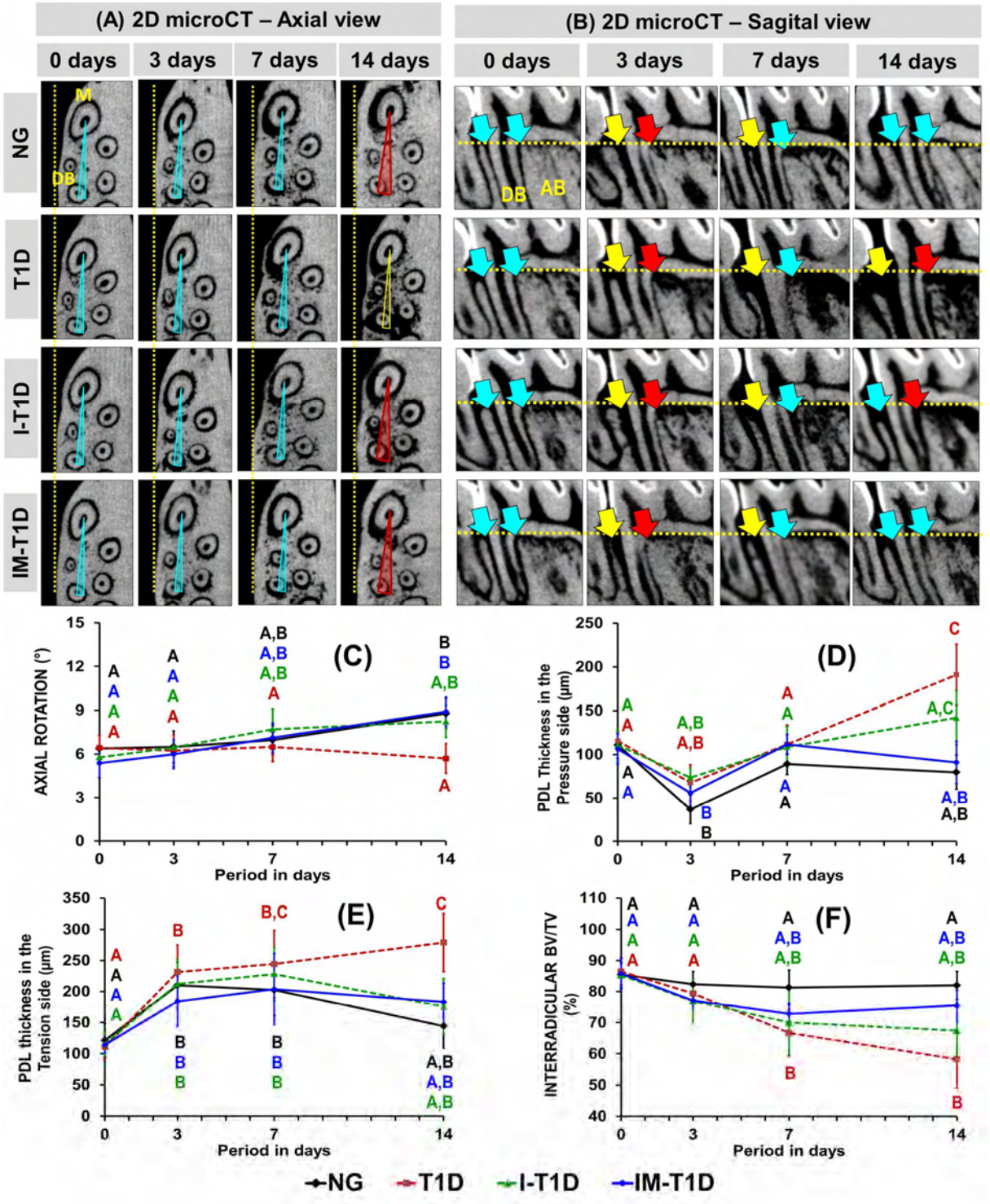
(A) 2D microCT – Axial view
(B) 2D microCT – Sagital view
0 days
3 days
7 days
14 days
NG
T1D
I-T1D
IM-T1D
M
DB
AB
(C)
AXIAL ROTATION (°)
(D)
PDL Thickness in the Pressure side (µm)
(E)
PDL thickness in the Tension side (µm)
(F)
INTERRADICULAR BV/TV (%)
Period in days
NG
T1D
I-T1D
IM-T1D

Figure 3

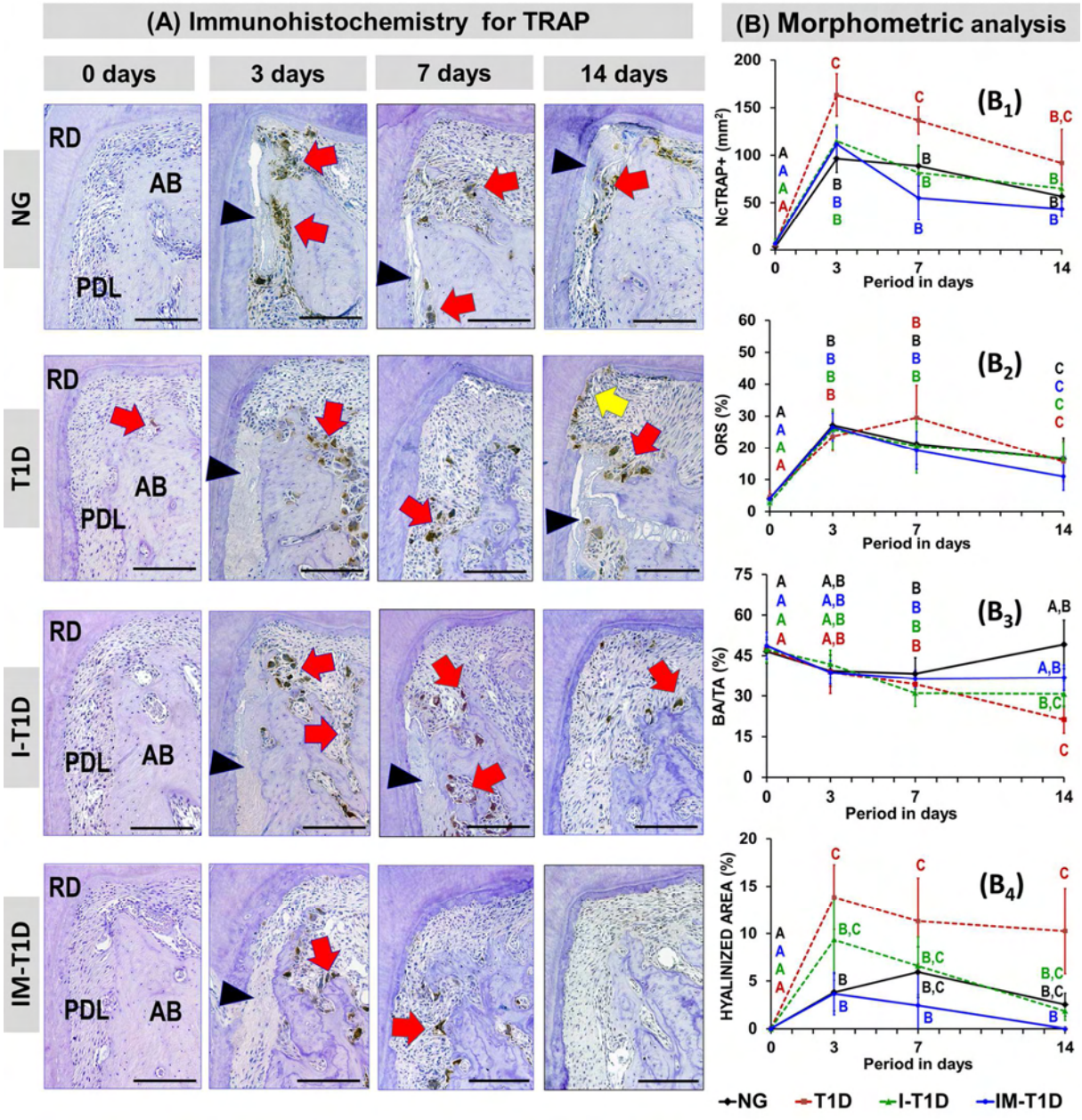
(A) Immunohistochemistry for TRAP
0 days
3 days
7 days
14 days
NG
T1D
I-T1D
IM-T1D
RD
AB
PDL
(B) Morphometric analysis
(B1)
NcTRAP+ (mm²)
(B2)
ORS (%)
(B3)
BA/TA (%)
(B4)
HYALINIZED AREA (%)
Period in days
NG
T1D
I-T1D
IM-T1D

Figure 4

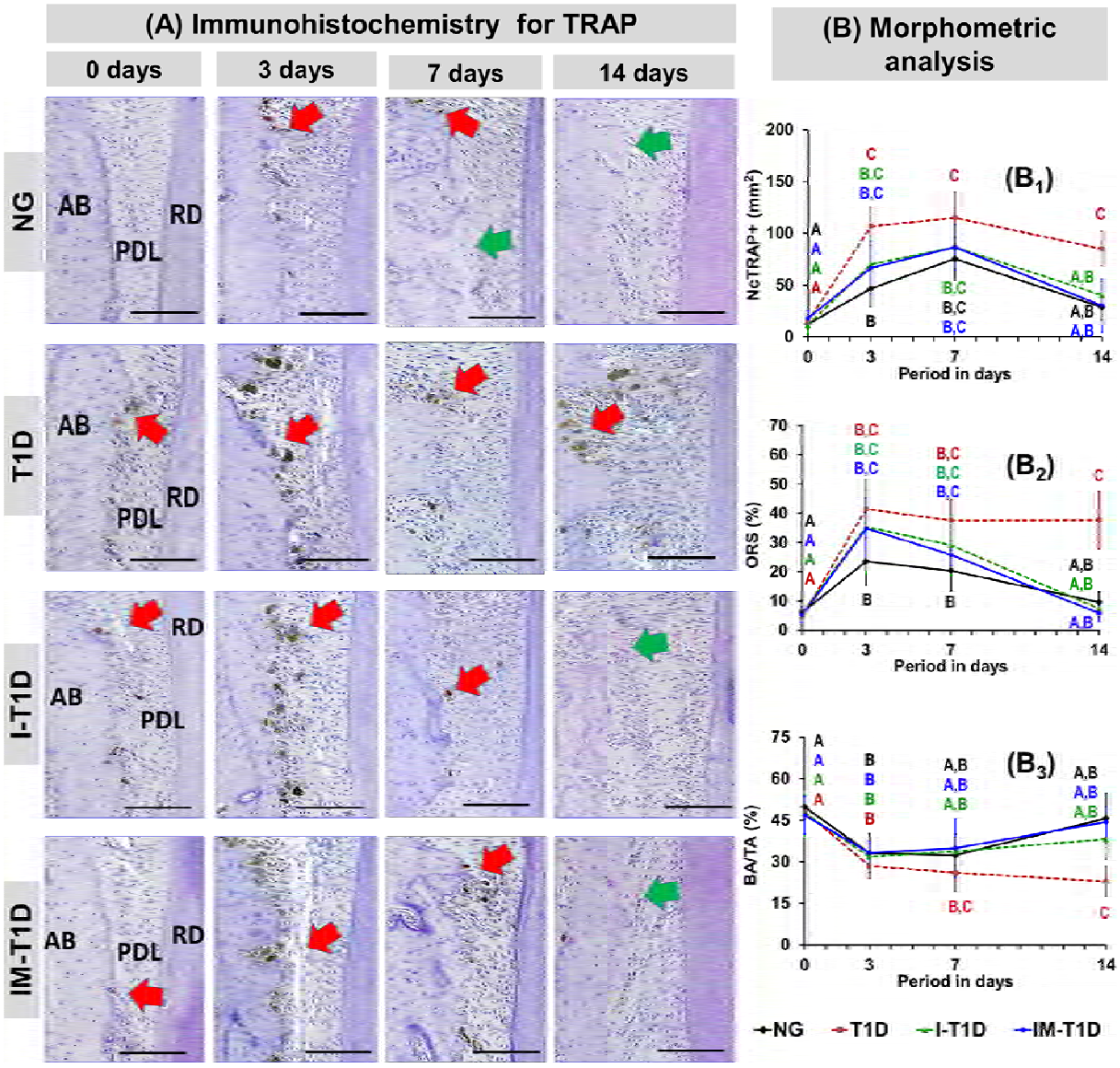
(A) Immunohistochemistry for TRAP
0 days
3 days
7 days
14 days
NG
T1D
I-T1D
IM-T1D
AB
PDL
RD
(B) Morphometric analysis
(B1)
NgTRAP+ (mm²)
(B2)
ORS (%)
(B3)
BA/TA (%)
Period in days
NG
T1D
I-T1D
IM-T1D

Figure 5

2.2 ARTICLE 2 –

Metformin therapy to prevent periodontal breakdown after orthodontic forces in type two diabetic rats. A micro-ct, histomorphometric and immunohistochemical evaluation

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ABSTRACT

Aim: We aimed to study evaluate the effects of metformin (MET) on the periodontal response to orthodontic forces in type two diabetic (T2D) rats. **Materials and methods:** Eighty-four Wistar rats were divided in normoglycemic (NG, n = 28), type two diabetics (T2D, n= 28) induced by high fat diet and low dose of streptozotocin, and T2D treated with MET 150mg/kg (M-T2D, n = 28). T2D was confirmed by fasting blood glucose ≥ 180 mg/dL. Glucose tolerant and insulin resistance tests were also conducted. After 25 days of T2D induction, orthodontic mesial tooth movement (OTM) was conducted for periods of 0, 3, 7, and 14 days. Samples were analyzed by micro-CT, histomorphometry and immunohistochemistry for TRAP. **Results:** Alveolar bone volume fraction (BV/TV and BA/TA) is reduced in T2D versus M-T2D and NG before OTM. During OTM, significant higher OTM, mesial tipping, mesial rotation, intrusion, periodontal thickness spacing and loss of alveolar BV/TV and BA/TA was found in T2D versus M-T2D and NG. It is accompanied by higher resorption areas and TRAP cells number on pressure side. **Conclusion:** Metformin therapy prevents from T2D increased osteoclastic resorption activity, reduced alveolar bone density, periodontal spacing and altered tooth movement patterns during orthodontic tooth movement.

1. Introduction

Diabetes mellitus represents a spectrum of metabolic disorders characterized by chronic hyperglycemia, a direct result of altered insulin secretion, insulin action, or both (American Diabetes, 2009; Yamamoto et al., 2018). Diabetes is rising to alarming epidemic levels. Current global prevalence of diabetics is about 425 million people and 90% of them are Type 2 (T2D) (IDF, 2019). T2D is a 2 hit disease that includes an impaired insulin secretion through pancreatic β-cell dysfunction and impaired insulin action through insulin resistance (Yamamoto et al., 2018). T2D, most commonly present in older adults, is now increasing in children, adolescents and younger adults because of the rising obesity levels, physical inactivity and poor diet. Obesity is the most potent risk factor for T2D, it contributes to insulin resistance by elevating the levels of circulating free fatty acids, which, in turn, inhibit glucose uptake, glycogen synthesis and glycolysis (Cerf, 2013; Goyal & Jialal, 2019). The American Diabetes Association recommends screening for overweight in children and adolescence in order to detect T2D ("Type 2 diabetes in children and adolescents. American Diabetes Association," 2000)

In odontology, both T1D and T2D were reported to have higher risk to develop periodontal disease, and subsequently higher risk to have poor bone health and to lose their teeth (Bandyopadhyay, Marlow, Fernandes, & Leite, 2010; Salvi, Carollo-Bittel, & Lang, 2008; Wu, Xiao, & Graves, 2015). At this point T1D and T2D share some similarities due to hyperglycemia, nonetheless both have inherent and different factors (e.g. ranging age, obesity and insulin resistance) that contributes differently in the periodontal breakdown pathogeny and course (Dursun et al., 2016). As periodontium appears brittle under diabetic state, other dental procedures might also be affected. More specifically, orthodontic studies related to

diabetes are scarce. Accordingly, uncontrolled hyperglycemia, in young T1D and young adult T2D animal models, exacerbates periodontal response to orthodontic forces leading to an up-regulation of osteoclast activity and down-regulation of osteoblast. These events eventually result in greater orthodontic tooth movement and teeth misalignment (Braga et al., 2011; Holtgrave & Donath, 1989; Mena Laura et al., 2019; Najeeb et al., 2017; Plut et al., 2015; Sun et al., 2017). According to (Chauhan et al., 2018) to prevent from detrimental diabetic effects during orthodontics, important care such as force loading, medication and systemic condition (hypo or hyper glycemia) have to be part of the orthodontic treatment approach. Diabetes itself does not represent a contraindication to orthodontic therapy, but uncontrolled diabetes entails negative consequences to the treatment (Patel, Burden, & Sandler, 2009).

First therapy for T2D is based on metformin (MET). It has effective glucose-lowering effects, relatively low cost, few side effects, and absence of weight gain. In this regard, important organizations of endocrinology and diabetes from America and Europe agree on recommending early initiation of MET as a first-line drug for monotherapy or combination therapy for T2D. (Hostalek, Gwilt, & Hildemann, 2015; Lily & Godwin, 2009; "Summary of revisions to the 2011 clinical practice recommendations," 2011). Metformin is also associated with short-term weight loss, improvement of insulin sensitivity, and decreased visceral fat (Hostalek et al., 2015; Tock et al., 2010). In odontology, MET is been used topically as an adjunctive therapy for better periodontal recovery during periodontal treatment (Pradeep et al., 2017). With respect to periodontium under orthodontic forces, in a previous study, we evaluated MET use as an adjunctive therapy for insulin in T1D young rats undergoing OTM. Besides a better glycemic control, MET addition significantly reduced the loss of alveolar density, PDL spacing, and controlled the altered tooth movement pattern (Mena Laura et al., 2019). Moreover, (Sun et al., 2017) recently pointed that MET reverses

the adverse effects of T2D during orthodontic tooth movement, and outcomes are supported by histologic analysis.

Among different T2D animal models, induction by high fat diet (HFD) and streptozotocin administration simulates T2D onset similarly to that of humans because it develops obesity, insulin resistance and hyperglycemia (Gheibi, Kashfi, & Ghasemi, 2017). Additionally, HFD-induced diabetic rats in laboratory develops osteoporosis and periodontal breakdown similarly to that of T2D patients (Ham, Choi, Lee, & Lee, 2019). In this study we hypothesized that MET has a protective effect form periodontal breakdown in type 2 diabetes after orthodontic forces application in T2D rats induced by HDF and Streptozotocin. Tooth movement patterns and tissues changes were approached by micro-CT histomorphometry and immunohistochemistry for TRAP cells.

2. Materials and methods

Experimental design, groups and diabetes induction (Fig 1)

Experimental procedures were approved by Animal Care Committee of Bauru Dental School of Sao Paulo University, research protocol CEEPA 9/2014. Eighty-four male 90 days old Wistar rats weighing 425g ±25g were housed under controlled light/dark cycle (12/12h) and temperature conditions with free access to food and water. Animals were randomly divided in normoglycemic (NG, n = 28) receiving normal diet (Presence Nutrition Animal, Paulínia-SP, Brazil), type two diabetics (T2D, n = 28) receiving high fat diet HFD for 90 days and a single injection of streptozotocin 23mg/kg (STZ) intraperitoneally (IP), and T2D treated by metformin (M-T2D, n = 28) receiving HFD, STZ and daily 150mg/kg of metformin by gavage 5 days after diabetes induction. HFD –modified from AIN-93M diet- has 59% fat, 11% protein and 30% carbohydrate from total kcal (Farhangi, Nameni, Hajiluian, & Mesgari-Abbasi, 2017).

Diabetes confirmation, GTT and ITT

Prior to MET treatment and tooth movement, T2D was confirmed by fasting blood glucose ≥ 180 mg/dL and its clinical characteristics at day -25. Additionally, a glucose tolerance test (GTT) and insulin tolerance test (ITT), were conducted at day 0 of OTM. Tests followed 6 hours of fasting, anesthesia with 60mg/kg thiopental sodium, and 2.0g/kg glucose IP injection for GTT or 2IU/kg regular insulin for ITT. In tail-tip blood samples, glycemia was measured at 0, 15, 30, 60, 90 and 120 min for GTT and at 0, 10, 20, 30 and 45 min for ITT respectively using a glucose-meter (Accu-chek Active Roche, Mannheim, Germany). Glucose areas under the curve (AUC) were calculated by trapezoidal approximation of glycemic levels. Glucose decay constant (Kitt) was calculated by the formula 0.693/ t1/2 (Bonora et al., 2000). The faster and more intense the glucose drop, the greater the insulin sensitivity (Geloneze, Tambascia, & UNICAMP, 2006)

Orthodontic appliance

Orthodontic appliance was installed according to (Mena Laura et al., 2019) with the aim of moving the upper first molar (M1) mesially. A 0.10 x 0.30 mm nickel-titanium closed coil spring producing 50g of force is attached to M1 and the incisors with a 0.08inch stainless steel (SS) ligature (see picture in Fig. 1).

Euthanasia of animals and collection of samples

At the end of experimental periods, rats were euthanatized by anesthetic overdose and the maxilla was carefully removed, hemisected and placed in 10% neutral buffered formalin. Samples underwent micro–computed tomography (micro-CT) scanning then demineralization in EDTA for immunohistochemical processing.

Micro-CT parameters and analysis (Figure 2A)

Sample scanning parameters in Skyscan micro-ct (Skyscan 1176 scanner and softwares, Bruker Skyscan, Aartselaar, Antwerp, Belgium) included: 80 kV voltage, 300 µA

current, 0.5 mm thick Al filter, 530 ms exposure time at 0.5° intervals and angular rotation of 180°. Images were 3D reconstructed using Nrecon software, aligned intersecting the sagittal, coronal and transaxial (palatal) planes at the center of the disto-buccal root (DBR) of the M1 in the Dataviewer software, and analyzed in the CTan software using previous reported parameters (Mena Laura et al., 2019). Measures (Fig. $2A_{2-6}$) included: mesial movement as the distance between interproximal M1 and M2 faces at their most convex point; vertical movement as the distance between palatal plane and furcation; periodontal thickness as the distance between DBR and crestal bone in the distal side, and interradicular bone in the mesial side; mesial tipping as the angle between palatal plane and mesial root (MR) axis line; axial rotation as the angle between a sagittal line and a line that crosses the center of de MR and DBR; palatal inclination as the angle between disto-palatal root axis and palatal plane; and interradicular bone volume fraction as Bone Volume over Total Volume (BV/TV), a 3D polyhedral area with 1,5mm depth obtained by drawing a polygonal Region of Interest (ROI) in about 150 consecutive scan slices.

Immunohistochemical laboratory procedures

Five maxillae from each experimental period were included in Histosec, then serial sagittal sections of 4µm of thickness at the DBR of the M1 were collected for TRAP (goat polyclonal anti-human peptide N-17, sc-30832) immunostaining. For immunolabeling, tissue sections were deparaffinized and immersed in 3% hydrogen peroxide to block endogenous peroxidase activity then washed with PBS and left in citrate solution for antigen retrieval. Afterward, serological proteins were blocked with a milk powder solution Molico 7% (Nestlé®) for 15 min. Then, the specific antibodies for each protein were 1/50 diluted in ADS-125 (Spring Bioscience) and incubated for 1 hour in a humid chamber at room temperature. Negative control cuts were incubated with diluent only. For color reaction or detection, cuts were incubated in N-Histofine (Simple stain Rat MAX PO NICHIREI

BIOSCIENCES INC.) for 30 minutes at room temperature. Finally, sections were washed and exposed to DAB solution (3,3'-diaminobenzidine, Dakocytomation) for 2-3 minutes and counterstained with Harris Hematoxylin for 1 minute.

Histomorphometry on pressure and tension sides

Morphological analysis was performed, in three slides per sample by a single observer using an image capture system composed by a camera AxioCam HRc coupled to a Microscope Axioscop 2 using 2X, 4X, 10X and 40X objectives and the software AxioVision 4.8 (Carl Zeiss, Oberkochen, Jena, Germany). For each slide, analyzes included: TRAP+ cells/mm^2, alveolar bone BV/TV, hyaline areas (HA), osteoclast resorption areas (ORS) in a total area (A) or region of interest (ROI) at the cervical and medial portion of the disto-buccal root on both pressure and tension sides (Fig. 2B).

Statistical Analysis

Quantitative data was submitted to Kolmorov Smirnov normality test and Bartlett's test for equal variances. Afterwards, data was submitted to one-way Analysis of Variance (ANOVA) for temporal evolution for each experimental group and different groups per period. For the variables that presented differences ($p < 0.05$), the Tukey's multiple comparison test was performed. All tests were performed using the GraphPad Prism 5 software (GraphPad Software, In. San Diego, CA, USA).

3. Results

Body mass (Fig. 3A) at 90 days of age averaged 421 ± 43.47g. After 90 days of HFD consumption T2D and M-T2D weight significantly increased 95% (821 ± 54.5g), whereas in NG increased 68% (708 ± 32.31g). During OTM body mass did not change significantly ($p > 0.05$).

Fasting plasma glucose (FPG) (Fig. 3B) at 90 days of age was similar among groups (58.1 ± 7.72 mg/dl). After 105 days of HFD consumption, FPG in T2D and M-T2D increased to 35.8% (78.9 ± 7.94mg/dl), whereas in NG it increased 10.4% (64.15 ± 5.43mg/dl). Five days after streptozotocin administration, FPG increased 281% (221.14 ± 58.76mg/dl) while in NG 26.6 % (to 81.6 ± 5.61mg/dl). During the remaining MET treatment days (-25 to 14), FPG in T2D remained constant (249.23 ± 16.15 mg/dL), and in NG it slightly increased (93.04 ± 9.2 mg/dl), however in M-T2D it significantly decreased to 134.72 ± 33.75 mg/dL, being statistically similar to NG at that point ($p>0.05$).

GTT, AUC, ITT and glucose decay constant (KITT)

Baseline glycemia of T2D (204.0 ± 50.4 mg / dL) was higher than NG (108.5 ± 8.0 mg / dL) and M-T2D group (153.8 ± 40.2 mg / dL) which were similar ($p> 0.05$). HFD for 120 days negatively influenced GTT (Fig. 3C) when compared to NG. MET treatment for 25 days favorably improved glucose tolerance and proximate to that of NG ($p> 0.05$). Differences among AUCs were NG = 19888 ± 1736, T2D = 48947 ± 7921 and M-T2D = 32927 ± 5594 (Fig. 3D). ITT and glucose decay rate KiTT % min (Fig. 3E-F) showed T2D (0.57 ± 0.19%) and M-T2DM (1.31 ± 0.14%. min-1) having lower insulin sensitivity than NG (1.71 ± 0.14% .min-1), however M-T2D sensitivity was higher than T2D ($p <0.01$).

Micro-CT

M1 positional changes during OTM are represented in Figs.3A-E and 4A and C. At day 0, all parameters were similar ($P\geq0.05$). Following orthodontic forces loading, a progressive M1 mesial movement (Fig. 4A-B) occurred along the experiment. At 3 days, in T2D (112.1±10.26 μm) and M-T2D (113 ±17.89 μm), displacement was higher than NG (81.9±11.61μm). It increased slightly until 7 days in all groups but insignificant ($P\geq0.05$). Then, at 14 days tooth displacement in M-T2D (144±28μm) and NG (122±26μm) was similar, however in T2D (240±31μm) it was in average 80.54% higher ($P=0.0068$). Regarding

vertical movement (Fig. 4A-C), NG and M-T2D did not change (P=0.6665 and P=0.1978, respectively) along periods, however a significant M1 intrusion of 293μm occurred in T2D (0.0053). A slight mesial tipping of 4º (P=0.012; Fig. 4A-D) and mesial axial rotation of 3º (P=0.0112; Fig. 5A and C) happened only in T2D at day 14. Palatal tipping was not recorded in all groups (Fig. 4A-E).

Periodontal changes along OTM are in Figs. 5B, E-F. Among groups, PDL thickness in both pressure (66.4±5.43μm; P=0.6853) and tension sides (76.7±4.33 μm; P=0.5797) were similar at day 0. On pressure side, only NG showed significant reduction at 3 days that remained constant until 14 days, on the other hand, in T2D and M-T2D a only similar slight reduction occurred at 3 (mean of 52.9±6.88μm) and 7 days (mean of 46±17.2μm) however insignificant (P≥0.05). On tension side, a gradual PDL thickness increase was observed until 7 days in all groups (117.3±20.21μm for NG; 193.5±59.29 for T2Dμm and 154.7±45.39μm for M-T2D). Between 7 and14 days, PDL thickness presented a slight tendency of reduction in NG and M-T2D (mean of 135±39.2μm), while in T2D it increased 37% (265.2±60.08μm).

Interradicular 3D bone volume fraction (BV/TV; Fig. 5Aand D) of T2D and M-T2D were 10.5% lower than that of NG at day 0 of OTM (75.8±6.07% and 75.7±4.49% vs 84.6±3.16%, respectively; P=0.0168). During 14 days of OTM, BV/TV did not show significant changes in NG (mean of 78.6±4.59%; P=0.0937), while that of T2D and M-T2D gradually reduced, respectively, 25.1% (P=0.0005) and 15.05% until day 7 (P=0.0116).

Morphologic and morphometric approach

Morphometric approach of periodontal tissues during OTM is represented in Fig 6 and 7.

On pressure side, alveolar bone appeared less compact with more spaces in T2D and M-T2D versus NG at day 0 (BA/TA of 57.9±4.27%, 61±4.27% vs 66.6±4.6%; P=0.0196; Fig.

6A and B1). At day 3 alveolar bone surface approximated the dental root, narrowing PDL thickness, and forming hyalinized and reabsorption areas (Fig. 6A and B4 and 2). Although TRAP+ cells (Fig. 6A and B3) increased in all groups (4±1.67 cells/mm^2 at day 0 to 39.2±8.46 cells/mm^2), ORS was higher in T2D and MT2D than NG (22.5±3.55% and 20.4± 3.23 versus 16.4±3.47 %; P=0.0426). It was associated to a lower BA/TA in T2D and M-T2D (mean of 55.5±4.82%) than NG whose BA/TA increased to 75.9±5.38% (P≤0.0001). Between 3 and 14 days TRAP+ cells number and ORS significantly diminished in NG (21.3±5.9 cells/mm^2 and 9.7±2.85% respectively) and M-T2D (22.96±4.73 cells/mm2 and 11.5±1.76%, respectively) associated to a BA/TA recover proximate to that of day 0. In T2D Both TRAP+ cells and ORS remained high (37.4±8.43 cells/mm2 and 19.6±5.08%, respectively) leading to a BA/TA decrease (45.7±6.58%). Hyaline areas (Fig. 6A and B4) appeared since day 3 in all groups however those persisted only in up to 7 days, being higher in NG (10.6 ± 2.56%; P=0.035) versus T2D and M-T2D (mean of 4.9±2.3%).

On the tension side at day 0, in all groups, the alveolar bone crest showed integrity with BA/TA (Fig. 7A and B1) of 47.3±5.04% (P=0.1008), and few TRAP+ cells (9.5±3.8 cells / mm^2; p=0.6469; Fig. 7A and B3) and ORS of 3.7±1.55% (P=0.1237; Fig. 7A and B2). At 3 days, in all groups, TRAP+ cells number (23.2±7.6cells / mm^2) along with ORS (12.2±3.7%) increased 1.43 times and 2.3 times respectively, leading to a BA/TA 0.39 times reduction (29.1±4.6%). Between 7 and 14 days, gradual recover or decrease of TRAP+ cells, ORS occurred in al groups, reaching similar values to that of day 0. Yet, in BA/TA, only NG and M-T2D recovered to an initial percentage (mean of 44.1±8.02%) while in T2D the recover did not reach initial values (34.6±6.49%).

4. Discussion

In the present study, HFD combined with STZ produced pathognomonic signs of T2D such as obesity/body mass gain, insulin resistance, and inflammation (Fujita, Watanabe, & Maki, 2012; Gautam et al., 2014). MET treatment promoted significant glycemic control reaching NG values at the end of the experiments. T2D resulted in maxillary osteoporosis in a period prior to orthodontic movement. Force loading on cells and tissues produce osteoclastic alveolar bone reabsorption, bone formation, and periodontal tissues remodeling, necessary events for a successful orthodontic tooth movement (Nishijima et al., 2006). Following orthodontic force application, alveolar bone volume fraction decreased even more and gradually in all groups. We found that treatment with MET in T2D animals significantly reduced diabetic deleterious effects on periodontal tissues and improved alveolar bone density. Additionally, MET favorably controlled tooth movement pattern, and cellular and tissue activity proximate to that of normoglycemic animals. Such tissue events during OTM in T2D have not yet been described in the literature.

In T2D animals, greater amount of tooth movement was observed versus NG and M-T2D. Although the appliance is designed to move M1 mesially, in T2D M1 intruded, tipped and rotated mesially. In this study the spatial or positional changes of M1 were approached by micro-CT, a very acute method to analyze tridimentionally hard and soft tissues (Hashimoto et al., 2013). T2D OTM studies are scarce, (Sun et al., 2017) using a similar model, are the first to report greater mesial movement in T2D-induced rats, by manual measurements made on silicone molds taken from jaws. It was different from (Plut et al., 2015), who, did not find OTM differences among T2D genetic model and control groups. Interestingly, approached by micro-CT, osteoporotic rats exhibit greater amount of tooth movement than control animals (Hashimoto et al., 2013; Xu, Zhao, Xu, & Ding, 2013), and by micro CT, they showed higher

mesial inclination and tooth extrusion (Xu et al., 2013). In a previous study of our group, untreated T1D animals showed greater mesial movement, tipping, rotation and tooth extrusion (Mena Laura et al., 2019). A greater osteoclastic activity and bone resorption of the alveolar bone (Salazar et al., 2015) form a space that facilitates tooth displacement. Contrarily, when osteoporosis is treated, tooth movement is reduced by the osteoclastic activity inhibition (Hashimoto et al., 2013; Irin Sirisoontorn et al., 2011; I. Sirisoontorn et al., 2012). A similar effect, less tooth displacement, is seen in T1D after treatment by insulin (Braga et al., 2011) or metformin (Mena Laura et al., 2019). Regarding T2D, MET showed to reduce tooth movement rate (Sun et al., 2017). Tooth intrusion observed in T2D, suggest that even using the same model, it exists a variability among orthodontic appliances in OTM studies, leading to different directions besides mesial.

Those events were accompanied by higher PDL spacing in both pressure and tension sides of OTM of T2D animals. Such changes have not been reported. Accordingly, large PDL thickness during OTM is also found in osteoporosis (Xu et al., 2013) and T1D animals (Mena Laura et al., 2019). PDL thickness is not commonly reported in OTM studies and it is still a limitation while approaching periodontal tissues remodeling. In fact, PDL thickness variances give a more specific panorama of what is occurring within de alveolar bone volume and remodeling – i.e. a large PDL thickness during OTM points a clear disequilibrium between bone formation and resorption rates along with their related cellular activity. Additionally, this higher spacing points a lack of a lag phase for bone to form and fill the spaces left in the tension area (An, Li, Liu, Wang, & Zhang, 2017; Xu et al., 2013) also a higher resorption level on pressure side. It can well explain why BA/TA of T2D did not recover to initial values as NG and M-T2D. Eventually, MET therapy reduced PDL thickness spacing in both pressure and tension sides.

Following OTM, T2D evidenced a significant interradicular alveolar bone volume loss, and higher ORS and TRAP+ cells. This loss is similar to what happens when excessive or heavy forces are applied, an “undermining resorption" pattern (Profitt, 1994), On this regard, (Plut et al., 2015), in Goto-Kikasaki T2D animals also described lower BV/TV by histomorphometry after OTM and strong expression of osteogenic factors. In this study, the lower inter-radicular BV/TV was attenuated after MET treatment. However, in the histomorphometric approach, MET significantly improved BA/TA reaching NG values. In this regard, histomorphometry is more sensible than micro-ct while approaching bone resorption and osteoid deposition areas during a dynamic bone turnover (van 't Hof, Rose, Bassonga, & Daroszewska, 2017). OTM is susceptible to forces magnitude, health status, and medication. In addition, there is no consensus on whether MET increases bone formation or density in T2D users (Gao, Li, Xue, Jia, & Hu, 2010; Molinuevo et al., 2010; Sedlinsky et al., 2011; Shah, Kola, et al., 2010), or even if MET interferes significantly in bone metabolism (Jeyabalan et al., 2013; Jeyabalan et al., 2012; Zinman et al., 2010). Interestingly, PDL hyaline areas persisted up to 7 days only in NG without bone volume loss or ORS. This persistence may suggest a major bone resistance from resorption while soft PDL is more brittle or less resistant to mechanical forces.

Our results support for the protective effect of metformin against diabetic detrimental effects om periodontal tissues during orthodontic movement. Metformin is pointed to stimulate bone formation in vivo and in vitro based on AMP-activated protein kinase (AMPK) signaling in osteoblasts (Shah, Bataveljic, et al., 2010; Shah, Kola, et al., 2010). Accordingly, in our study, in T2D receiving MET, BA/TA on tension side was restored to initial values and this bone formation lead to PDL thickness reduction. Interestingly, bone significant bone formation is not seen in NG individuals receiving metformin (Jeyabalan et al., 2013). NG individuals receiving MET was not among our aims and, it still represents one

limitation of this study. Metformin is also pointed to avoid bone resorption by reducing osteoclastic activity (Feng, Liu, & Liu, 2012; Mai et al., 2011). Number of TRAP + cells was reduced in M-T2D animals on both pressure and tension sides. Similarly, Sun et al., 2017 pointed MET treatment to reduce TRAP + cells, on the pressure side. Metformin inhibits AMPK and CaM kinase kinase (CaMKK) by suppressing endogenous secretion of RANKL then osteoclasts activity in a dose-dependent manner. On the other hand, on osteoblasts, metformin activates AMPK and RUNX-2 in a time-dependent manner (Jang, Kim, Lee, Son, & Koh, 2011). Such molecules are still study targets for a better approach in the treatment of T2D and osteoporosis. Diabetes represents a systemic disorder of multiple etiology and effects, however, studies regarding tooth movement and periodontal tissues in such conditions are scarce. Specific T2D studies are still necessary to understand, then properly conduct the orthodontic therapy caring of its periodontal implications. The present study highlights protective effects of MET on deleterious effects on periodontal tissues during OTM in T2D model induced by HFD plus STZ.

5. Conclusions

HFD plus STZ in rats resembles T2D development similar to that of humans. It resulted in obesity, insulin resistance and maxillae osteoporosis prior to OTM. During OTM, T2D results in an increase of TRAP cells, resorption areas and reduced bone volume fraction, leading to altered tooth movement patterns. Metformin therapy achieves glycemic control, and tooth movement pattern proximate to that of normoglycemic individuals. Besides, it is accompanied with less bone loss and periodontal spacing.

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Conflicts of interest

All authors declare that there are no conflicts of interest, financial or otherwise, with respect to this study.

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FIGURE LEGENDS

Figure 1: Time line representation of the experimental design. From days -120 to -25 (90 days), during type two diabetes induction period, high-fat diet was administered and it culminated with a single injection of streptozotocin 23mg/kg at day -30. Afterward (day -25), daily metformin treatment started and lasted until the end of orthodontic tooth movement periods (P_0, P_3, P_7 and P_{14}). In picture, orthodontic appliance model to move rat molar mesially.

Figure 2: Micro-CT, histomorphometric and immunological approach (A and B): A1) 3D view of the upper right molars (M1, M2 and M3 respectively) and blue rectangle highlighting the general region of interest (ROI). Within the ROI, linear measurements include mesial and vertical displacement, and periodontal thickness on tension or pressure side (A2). Angular measurements (yellow angles) include mesial tipping (A3), palatal tipping (A4) and axial rotation (A5). 3D alveolar bone volume fraction (BV/TV) (A6) is reconstructed from several polygonal ROIs drowned in 150 consecutive 2D slices (blue ROI in A5). B) TRAP immunostained slide showing selected ROI in tension (B2) and pressure (B3) sides of the M1 distobucal root (B1). Parameters included: number of osteoclasts per square millimeter (NcTRAP+/mm2), osteoclast resorption surface percentage (ORS) or bone surface in contact to osteoclasts (sum of yellow lined **surfaces** S1+S2..+S5 per the total blue lined **surface length** X 100), and bone (yellow; BA/TA) and hyaline (red) area densities over the total area (green dotted line area).

Figura 3: Clinical data, mean ± SD of 5 samples: Body mass (A), glycemia (B), glucose tolerant test or GTT (C) AUC or area under de curve (D) of GTT, or insulin tolerance test (ITT) and ITT constant (KITT). Different letters indicate $p<0.05$ by one-way ANOVA.

Figure 4 3D micro-CT representative sagittal images (A) and graphics of linear (B and C) and angular (D and E) measurements. Greater M1 mesial displacement of T2D and M-T2D versus NG appeared since early periods (3 and 7 days), however at 14 days it is higher only in T2D. Note the major distance between M1 and M2 in T2D at day 14, besides perceive that M2 moves forward, accompanying M1 displacement days 7 and 14 (red arrows). Additionally, T2D displays significant tooth intrusion (C and blue dashed line in A) and mesial tipping (D and red angle in A) at day 14. No palatine tipping is observed (E). A reduction in alveolar bone level (yellow dashed line) in T2Dis evident. In the graphics, bars represent DPM and different letters indicate $p<0.05$ by one-way ANOVA.

Figure 5: 2D micro-CT representative axial (A) and sagittal (B) images and graphics of axial rotation (C), interradicular BV/TV (D) and PDL thicknesses (E and F). T2D shows higher mesial rotation (red angle in A) at day 14 than days 0 and 3. PDL thickness of the disto-buccal root DB (E and F) started similar in all groups at day 0 (blue arrow in B). On tension side (blue arrow) at day 3, it significantly increased in all groups and recovered (at day 14) totally in M-T2D and partially in NG, while T2D was significantly higher. On pressure side (red arrow)., at day 3 it reduced significantly only in NG remaining until day 14 where a partial reduction in M-T2D and no reduction in T2D is seen. Lower interradicular BV/TV (D) in T2D and M-T2D than NG is evident at day 0. Then, a gradual BV/TV significant reduction is observed in T2D and M-T2D until day 7, however at 14 days, in M-T2D BV/TV recovered but in T2D decrease. In graphics bars represent DPM and different letters indicate $p<0.05$ by one-way ANOVA.

Figure 6: Photomicrography of slides immunolabeled for TRAP (A) and graphics for BA/TA (B1), ORS (B2), NcTRAP+ (B3) and hyalinized areas (B4) on pressure side. At day 0, BA/TA was lower in T2D and M-T2D than in NG and continued until day 3. From day 7 to 14, BA/TA is recovered in M-T2D however in T2D it decreased gradually. ORS (red arrows) started similar among groups an increased at day 3, however it is higher in T2D than M-T2D and NG. From day 3 to 14, it

occurs an ORS gradual decrease in M-T2D and NG, while in T2D it remains high. Accordingly, TRAP cells Nº started similar and showed a pick at 3 days, decreasing gradually in NG and M-T2D, while in T2D, Nº remained high. Hyalinized areas (yellow arrows) formed at day 3 and decreased until day 14 in T2D and M-T2D. In NG these hyalinized areas persisted until day 7, then reduced at 14 days. Histological figures were captured with a 40X objective, the scale bar is of 100 µm and D = dentin. In the graphics, bars represent DPM and different letters indicate $p<0.05$ by one-way ANOVA.

Figure 7: Photomicrography of slides immunolabeled for TRAP (A) and graphics for BA/TA (B1), ORS (B2), and NcTRAP+ (B3)) on tension side. Similar BA/TA among groups reduced significantly at day 3 and 7 in NG and M-T2D, and recovered until day 14. In T2D, it significantly decreased until day 7, and partially recovered until day 14. In all groups, ORS (B2) and TRAP cells Nº (B3) showed a peak at 3 days and a gradual decrease returning to initial values at day 14. Figures were captured with a 40X objective, scale bar value is 100 µm and D = dentin. In graphics, bars represent DPM and different letters indicate $p<0.05$ by one-way ANOVA.

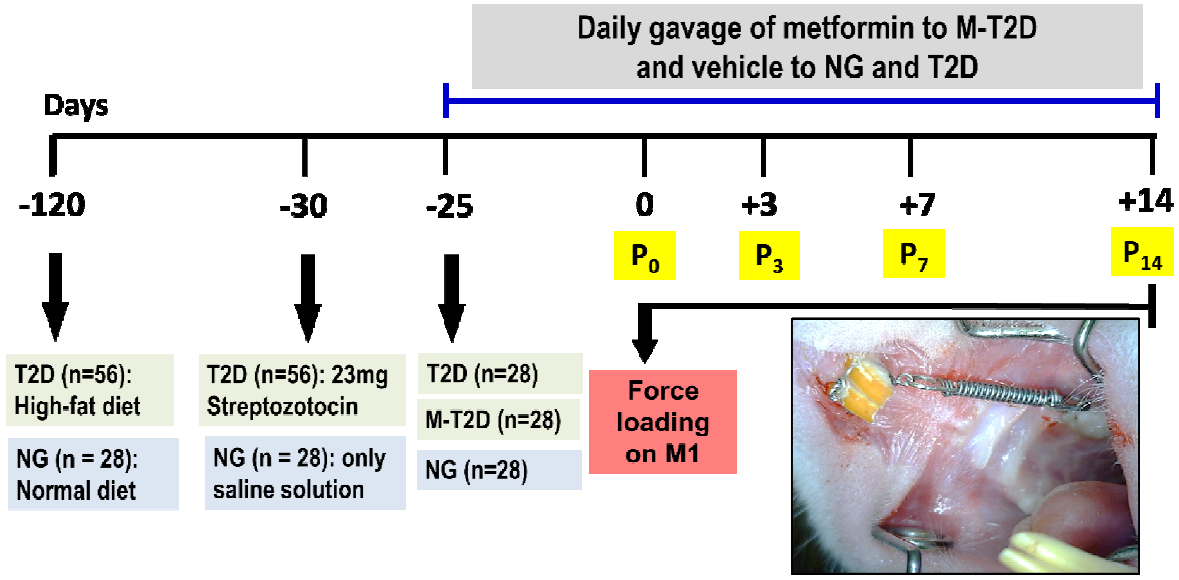

Figure 1:

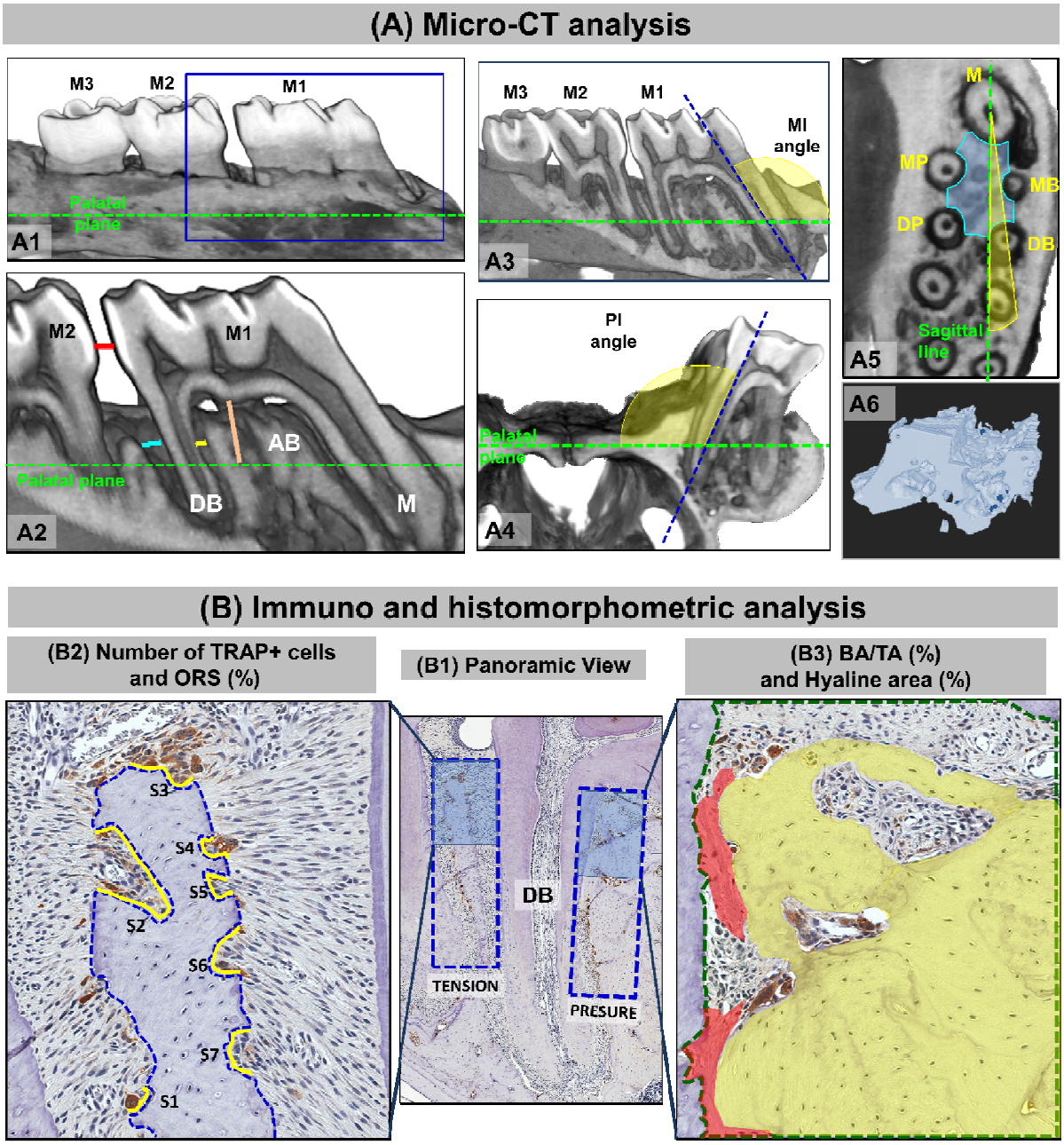
(A) Micro-CT analysis
M3
M2
M1
Palatal plane
A1
MI angle
A3
M
MP
MB
DP
DB
Sagittal line
A5
M2
M1
AB
Palatal plane
DB
M
A2
PI angle
Palatal plane
A4
A6
(B) Immuno and histomorphometric analysis
(B2) Number of TRAP+ cells and ORS (%)
(B1) Panoramic View
(B3) BA/TA (%) and Hyaline area (%)
S3
S4
S5
S2
S6
S7
S1
DB
TENSION
PRESURE

Figure 2:

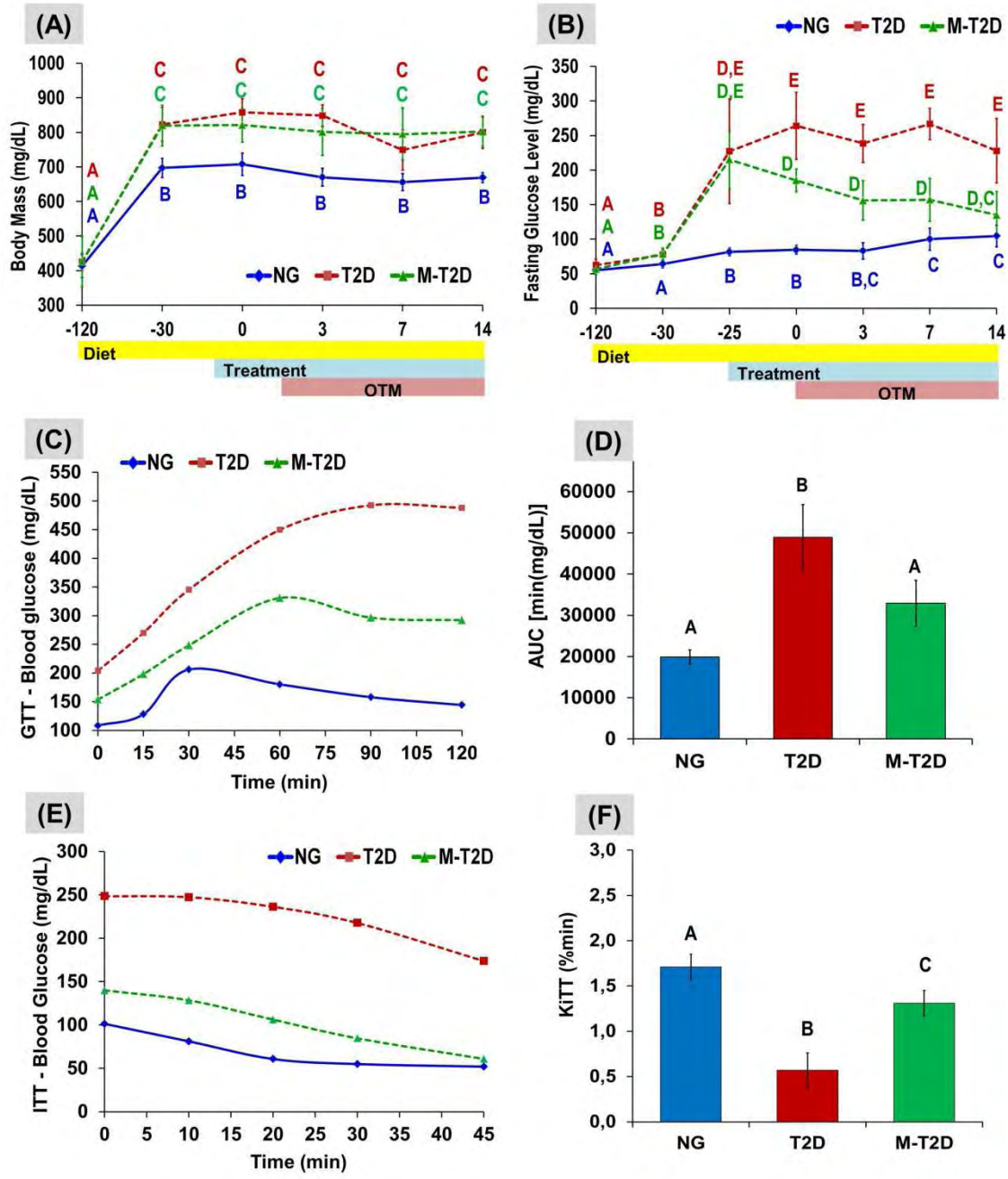
(A)
Body Mass (mg/dL)
NG T2D M-T2D
Diet
Treatment
OTM
(B)
Fasting Glucose Level (mg/dL)
NG T2D M-T2D
Diet
Treatment
OTM
(C)
GTT - Blood glucose (mg/dL)
NG T2D M-T2D
Time (min)
(D)
AUC [min(mg/dL)]
NG
T2D
M-T2D
(E)
ITT - Blood Glucose (mg/dL)
NG T2D M-T2D
Time (min)
(F)
KiTT (%min)
NG
T2D
M-T2D

Figura 3:

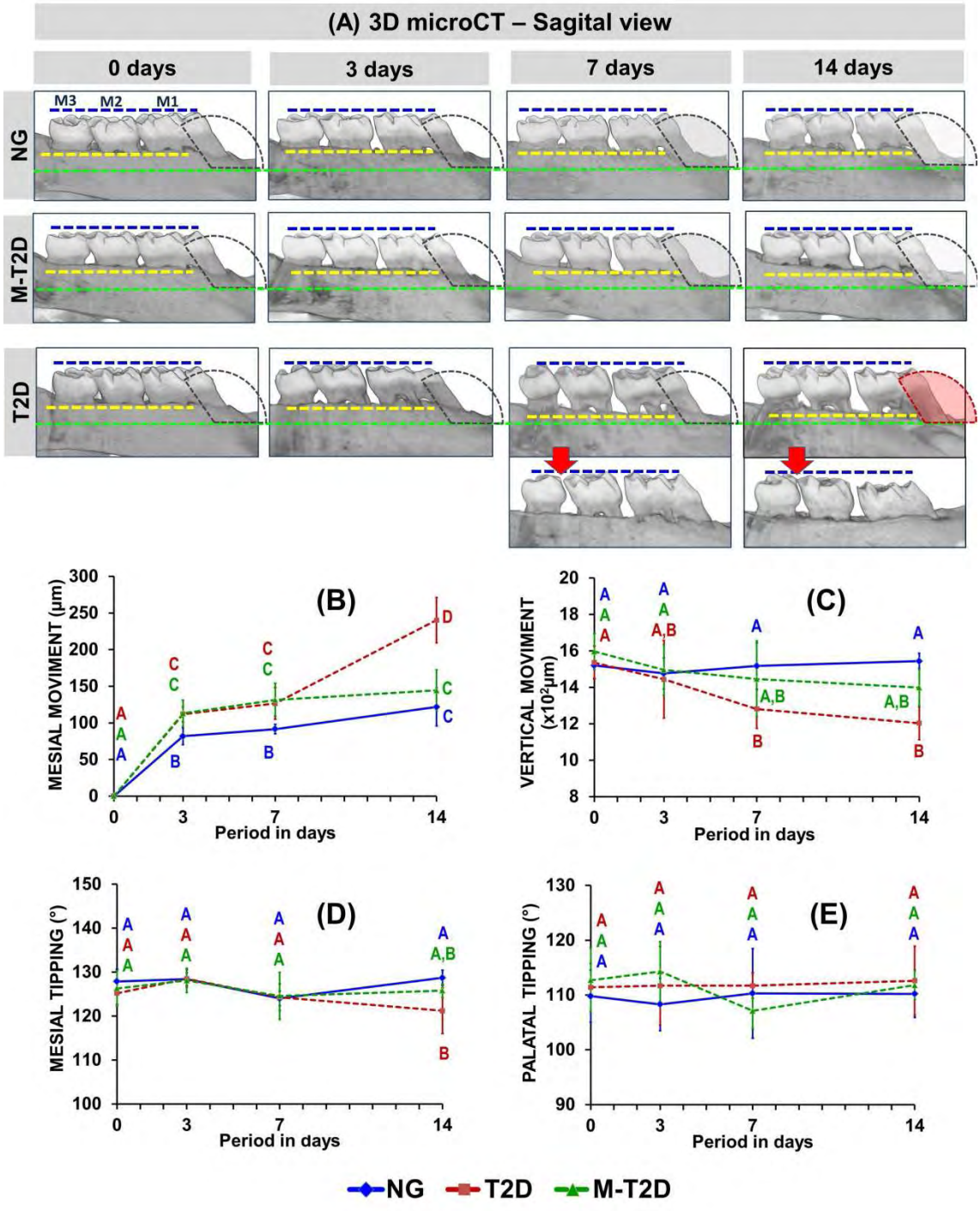
(A) 3D microCT – Sagital view
0 days
3 days
7 days
14 days
NG
M-T2D
T2D
M3
M2
M1
(B)
MESIAL MOVIMENT (µm)
Period in days
(C)
VERTICAL MOVIMENT (x10²µm)
Period in days
(D)
MESIAL TIPPING (°)
Period in days
(E)
PALATAL TIPPING (°)
Period in days
NG
T2D
M-T2D

Figure 4

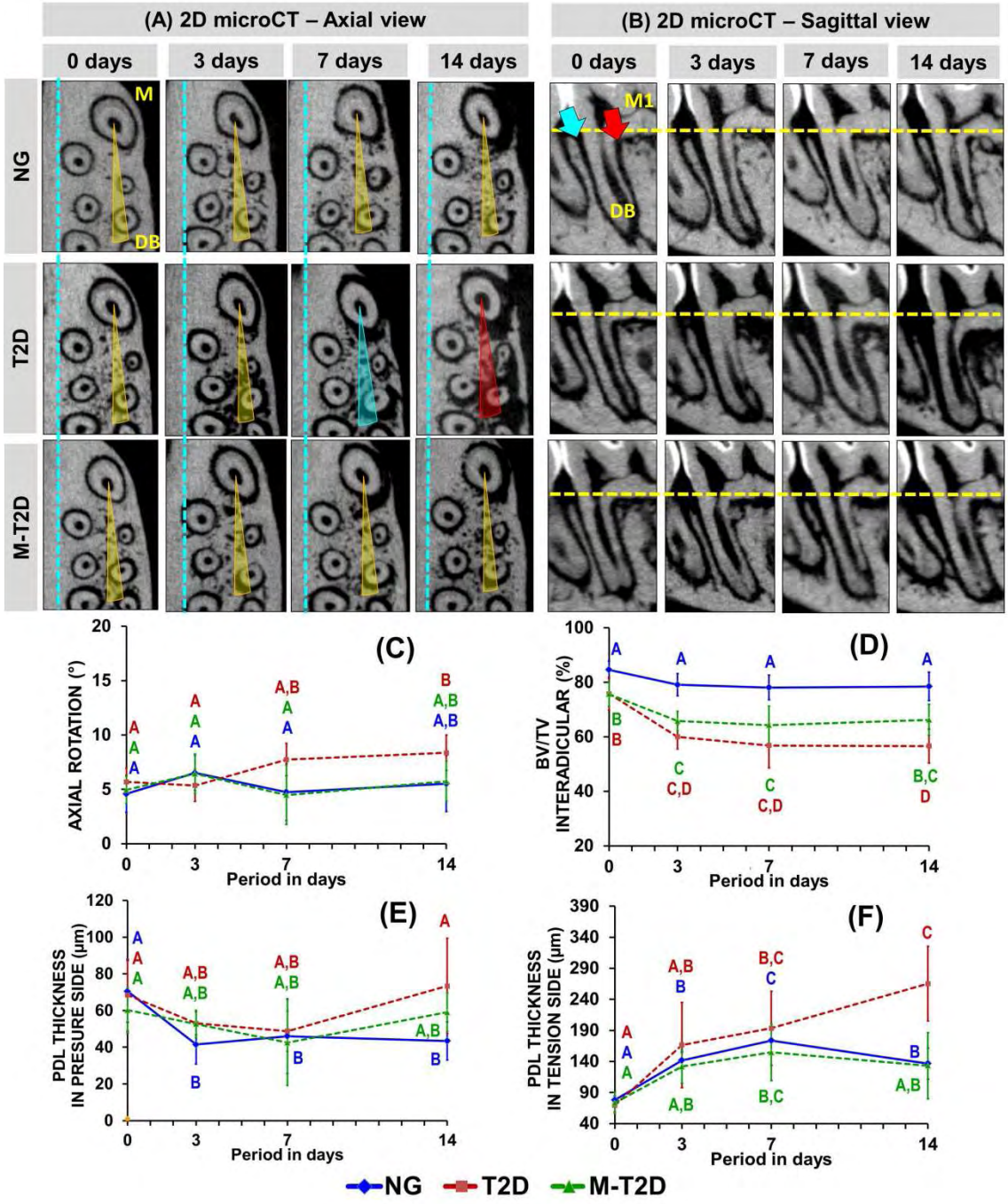
(A) 2D microCT – Axial view
(B) 2D microCT – Sagittal view
0 days
3 days
7 days
14 days
NG
T2D
M-T2D
M
DB
M1
(C)
AXIAL ROTATION (°)
Period in days
(D)
BV/TV INTERADICULAR (%)
(E)
PDL THICKNESS IN PRESURE SIDE (µm)
(F)
PDL THICKNESS IN TENSION SIDE (µm)
NG
T2D
M-T2D

Figure 5:

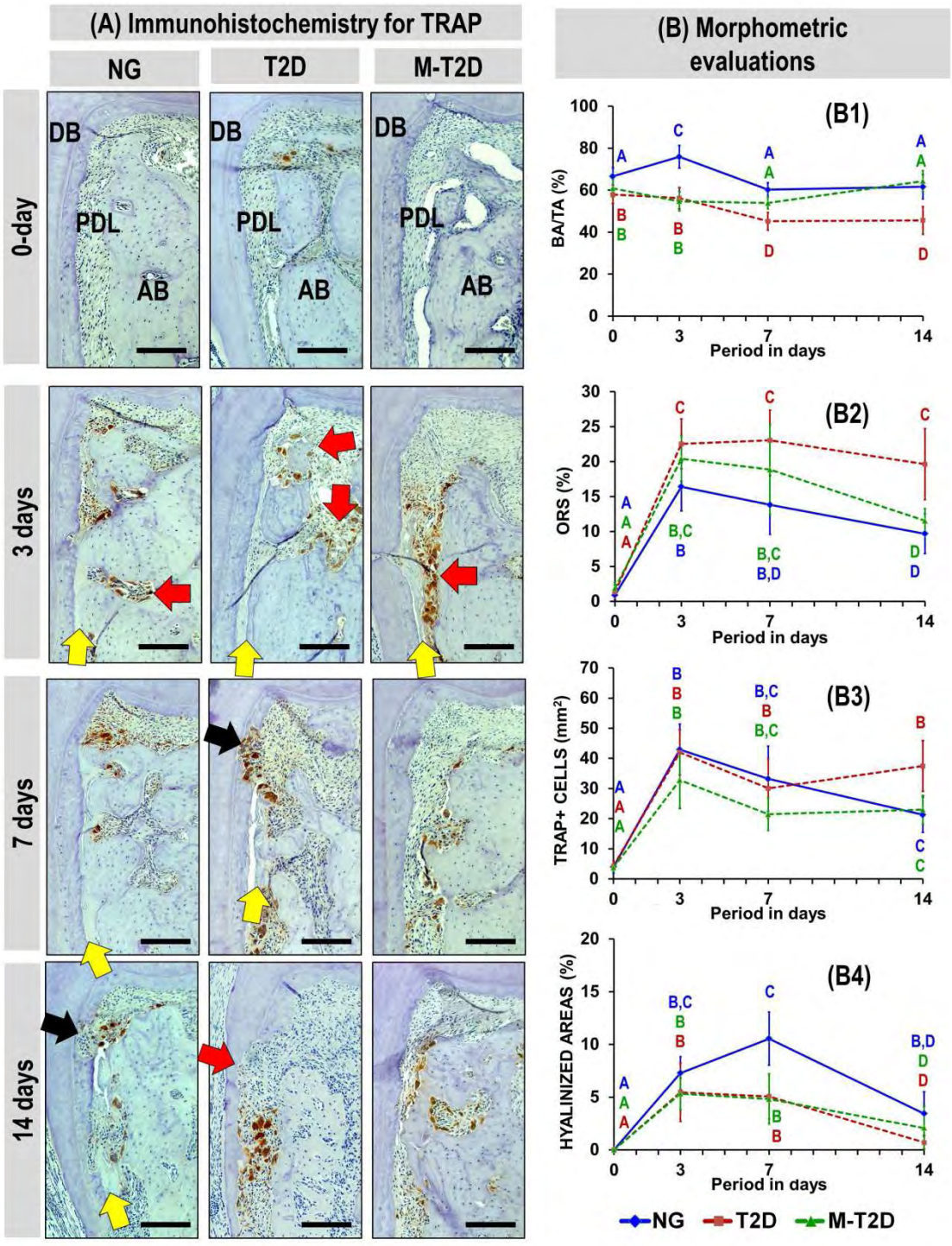
(A) Immunohistochemistry for TRAP
NG
T2D
M-T2D
0-day
3 days
7 days
14 days
DB
PDL
AB
(B) Morphometric evaluations
(B1)
BA/TA (%)
(B2)
ORS (%)
(B3)
TRAP+ CELLS (mm²)
(B4)
HYALINIZED AREAS (%)
Period in days
NG
T2D
M-T2D

Figure 6:

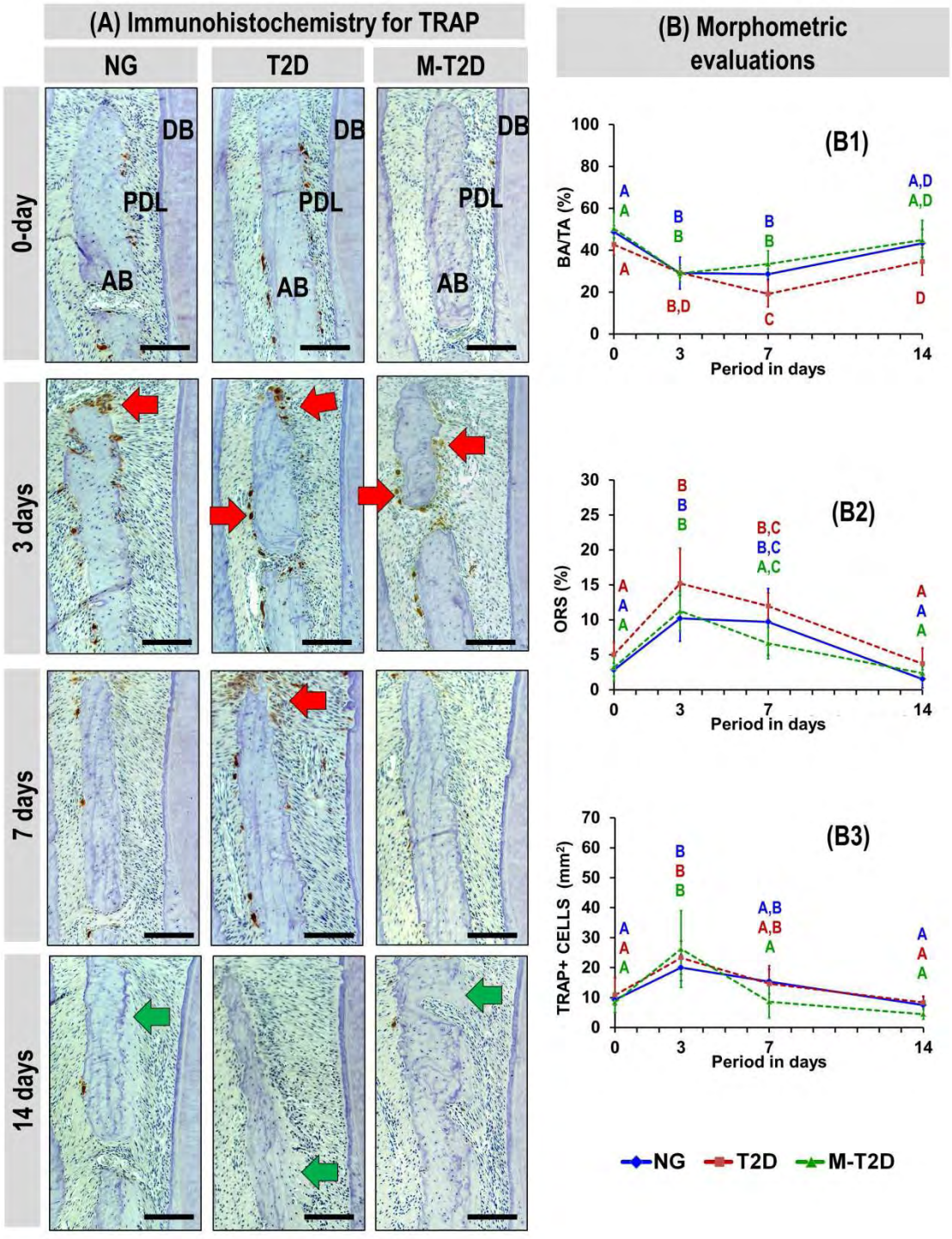
(A) Immunohistochemistry for TRAP
NG
T2D
M-T2D
0-day
3 days
7 days
14 days
DB
PDL
AB
(B) Morphometric evaluations
(B1)
BA/TA (%)
(B2)
ORS (%)
(B3)
TRAP+ CELLS (mm²)
Period in days
NG
T2D
M-T2D

Figure 7:

3 DISCUSSION

3 DISCUSSION

Metformin effects as and adjunctive therapy to insulin in T1D, and as single treatment in T2 was studied during orthodontic treatment. Force loading on cells and tissues produces osteoclastic reabsorption of the alveolar bone and remodeling of periodontal tissues, necessary events to produce successful orthodontic tooth movement (Nishijima *et al.*, 2006). In both studies we found that MET use promotes good glycemic control then protective effects on periodontal tissues undergoing tooth movement.

In this study we confirmed T1D and T2D model in rats displaying a different course of diabetes. On the one hand Streptozotocin administration to induce T1D lead to higher glycaemia (450 ± 105mg/dl), water and food intake accompanied by body mass reduction in a short period of time as it occurs in humans. Similar clinical adverse effects are pathognomonic of T1D individuals (Who, 2019). We found that MET adjunction to insulin significantly reversed this clinical condition better than insulin alone and close to that of NG individuals. On the other hand, HFD to induce T2D was administered for 105 days (from young adult age to adult age), and only after, a low dose of Streptozotocin was administered to slightly elevate glycaemia. In this type of diabetes, rats underwent body mass gain or obesity, insulin resistance and eventually hyperglycemia (less than 350mg/dl) just as T2D develops in humans (Carvalheira *et al.*, 2002; Srinivasan *et al.*, 2005; Cerf, 2013). In this study, T2D was significantly related to alveolar bone osteoporosis prior to orthodontic force application, accordingly, osteoporosis is a frequent T2D adverse effect (Anaforoglu *et al.*, 2009; Arikan *et al.*, 2012; Walsh and Vilaca, 2017). MET treatment efficiently controlled hyperglycemia achieving NG levels at the end of the experiments. Additionally, MET ameliorated the glucose tolerance and insulin tolerance since the beginning of treatment and it is also seen in current studies (Horakova *et al.*, 2019)). Significant alveolar BV/TV and BA/TA recover was only achieved at 14 days of OTM. In this context, whether MET benefit

osteoporosis or even reduces fragility to fractures in T2D remains controversial (Jeyabalan *et al.*, 2012; Mccarthy *et al.*, 2016). Nonetheless, this study supports for MET amelioration g alveolar bone density.

In normoglycemic animals, clinical parameters including body mas, water did not change significantly along the experiments. In the article 2, NG animals showed a small gradual increase of body mass until the end of the experiments, however it is concomitant to rat aging and entering to adult life (Nistiar *et al.*, 2012). During OTM the three tooth movement phases occurred: an initial phase with initial tooth displacement within the periodontal ligament, creating a widened tension side and a narrowed pressure side; a lag phase where no tooth movement is observed; and a post lag phase where a gradual accelerated tooth movement occurs (Krishnan and Davidovitch, 2006; Wise and King, 2008). Those phases where according to the experimental periods in our OTM model. At day 0, NG showed alveolar bone integrity, compact cortical with Sharpey fibers and a periodontal ligament composed of dense organized collagen fibers perpendicular / oblique to the surface of the alveolar bone, and richly cellular and vascular. PDL thickness appeared uniform, ranging from 35 to 90um, and BV/TV showed no alterations. At 3 days, *initial OTM phase*, higher PDL spacing and fibers stretching were evident on tension side, alveolar bone resorption also occurred. On the pressure side, alveolar bone surface was proximate to the dental root, narrowing PDL thickness and forming hyalinized and reabsorption areas. Between 3 and 7 days, a *lag phase* with almost no significant changes were observed. In the second experiment, hyaline areas persisted until day 7, still no BA/TA or BV/TV reduction was recorded or observed in both articles. Between 7 and 14 days, *post lag phase*, PDL underwent periodontal spaces recovery with reorganization of vessels and collagen fibers on both sides. On tension side, in NG it was accompanied with bone formation on alveolar bone surface. In some cases of NG (2/14) and M-T2D (3/14) and T2D (6/14) groups, root resorption

(cementum and dentin) were also evident. In this regard, root resorption is not evaluated in both articles and it may represent a limitation in both studies.

T1D significantly weakens periodontal tissues so that orthodontic forces lead to a high periodontal breakdown, associated to hyaline areas presence as it is observed in previous studies (Holtgrave and Donath, 1989; Villarino *et al.*, 2011). Besides, we found altered tooth movement pattern, periodontal spacing and loss of alveolar bone density. Such changes were not previously described. In the present study, MET addition to insulin clearly ameliorates clinical parameters of T1D animals. MET helps to regain body mass and to reduce glycaemia proximate to that of normoglycemic. Previous studies suggested that MET ameliorates insulin action in peripheral tissues then reduces insulin needs (Faichney and Tate, 2003; Hostalek *et al.*, 2015). However, controlling glycaemia in T1D remains difficult because of the risk of suffering hypoglycemic crisis (Sayarifard *et al.*, 2017) and coma among insulin users (Wright, 2003); therefore, different levels of hyperglycemia persist among T1D patients. Regarding OTM, the resulting periodontal breakdown is clearly prevented by MET addition. PDL showed to be brittle in such T1D condition, then as consequence, tooth movement pattern was altered. However, MET treatment prevented from those movements leading to a tooth movement pattern proximate to that of NG. MET addition also reduced the bone loss (BV/TV or BA/TA) observed in T1D. Whether MET has a direct effect on bone tissue is still under debate, however MET is related to higher osteoblastic activity among laboratorial studies (Jang, Kim, Bae*, et al.*, 2011; Jang, Kim, Lee*, et al.*, 2011; Mai *et al.*, 2011). In this regard, this study supports for MET having an osteogenic effect or at least related to higher alveolar bone volume fraction than insulin treated or untreated T1D.

Resembling T1D, T2D produced adverse effect on PDL tissues during OTM. The higher amount of tooth mesial displacement that occurs in both T1 and T2D versus NG, is not accompanied by bone formation in the tension side and just by tissues breakdown. Hence,

clinically, higher T1D or T2D tooth movement may represent a signal of unfavorable OTM in which adverse effects are happening. Orthodontic treatment planning studies regarding time of treatment and treatments goals to be achieved in diabetic patients are still scarce. Higher PDL spacing and OTM patterns is only been approached in an osteoporotic model study (Xu *et al.*, 2013). Some differences between T1D and T2D were found in the tooth movement pattern and alveolar bone density. The tooth intrusion instead of T1D extrusion may be explained by the brittleness of an osteoporotic alveolar bone in T2D (Parfitt, 2013). Accordingly, molar intrusion is been pointed as the most difficult tooth movement among orthodontics (Ayadi *et al.*, 2018). Osteoporotic bone entails higher turnover (Parfitt, 2013), thus, tooth intrusion were possible just because factors such as direction of the force, low resistance of tissues and high alveolar turnover gathered together in T2D. MET reduced the T2D deleterious effects on periodontium during OTM. Following orthodontic force loading, alveolar bone density continues to reduce along with periodontal spacing and an altered tooth movement pattern. However, in the MET treated group BV/TV as well as BA/TA recovered at 14 days. We found single MET treatment being enough to recover the detrimental effects of T2D on periodontal tissues subjected to OTM close to NG however most of these effects were seen at 14 days of OTM where MET therapy reached 39 days. Still molecular pathways are needed to understand cellular related activity. MET restoring bone loss after OTM in diabetics is supported by the PDL thickness reduction in tension side and BV/TV recovery.

4 CONCLUSIONS

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This study supports that:

- T1D and T2D have different harmful effects on periodontal tissues undergoing OTM.
- In T1D animals, force loading goes beyond the adaptive capacity of tissues. In addition, this causes cell death, extensive areas of tissue hyalinization and alveolar bone loss leading to a different tooth movement pattern.
- Metformin as an adjunctive to insulin therapy, promotes insulin sparing, better glycemic control and closer to NG values.
- MET plus Insulin avoids different tooth movement patterns and ameliorates the PDL response, reduces tissue damage and increases the PDL recovery better than insulin alone and similar to NG.
- HFD plus STZ in young adults wistar rats successfully resembled T2D development similar to that of humans; induction resulted in obesity, insulin resistance and maxillae osteoporosis.
- During OTM, T2D results in an increase of TRAP cells, resorption areas and reduced bone volume fraction, leading to altered tooth movement patterns.
- Metformin therapy achieves glycemic control, and tooth movement pattern proximate to that of normoglycemic individuals; it is accompanied with less bone loss and periodontal spacing.

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APPENDIXES

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article "**Metformin as an add-on to insulin improves periodontal response during orthodontic tooth movement in type 1 diabetic rats**" will be included in Thesis of the student **Ever Elias Mena Laura** was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, August 30, 2019.

Ever Elias Mena Laura
Author
Signature

Tania Mary Cestari
Author
Signature

Rodrigo Almeida
Author
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Daniela Santos Pereira
Author
Signature

Rumio Taga
Author
Signature

Gustavo Pompermaier Garlet
Author
Signature

Gerson Francisco Assis
Author
Signature

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article "**Metformin therapy to prevent periodontal breakdown after orthodontic forces in type two diabetic rats. A micro-CT, Histomorphometric and immunohistochemical evaluation**" will be included in Thesis of the student **Ever Elias Mena Laura** was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, August 30, 2019.

Ever Elias Mena Laura
Author
Signature

Luan Pereira Macena
Author
Signature

Ana Carolina Cestari Bighetti
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Daniela Santos Pereira
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Tania Mary Cestari
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ANNEXES

ANNEX 1

Approval of Ethical Committee article 1

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 033/2013

Bauru, 26 de maio de 2014.

Senhor Professor,

Em atenção às alterações no projeto de pesquisa denominado **Efeito da Metformina no osso alveolar durante a movimentação ortodôntica em ratos diabéticos induzidos pela Estreptozotocina,** de autoria de Ever Elias Mena Laura, com colaboração de Tania Mary Cestari e Danila Santos Pereira, sob sua orientação foi enviado ao relator para avaliação, quais sejam:

Alteração no título para: *"Influência de drogas antidiabéticas no metabolismo ósseo alveolar durante a movimentação dentária em modelos experimentais de ratos diabéticos tipo 1 e 2"*;

Número total de animais: 180 ratos, divididos em seis grupos experimentais, em 3 períodos experimentais.

Considerando que tais modificações não implicam em impedimentos éticos, o relator emitiu parecer favorável, o que foi aceito *ad referendum* desta Comissão.

Lembramos que qualquer outra alteração que ocorrer na pesquisa, esta Comissão deverá imediatamente comunicada, bem como ao final, um relatório com os resultados obtidos seja enviado para análise ética e emissão de parecer, o qual poderá ser utilizado para fins de publicação científica.

Atenciosamente,

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

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

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-101 – C.P. 73
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ANNEX 1

Approval of Ethical Committee Article 2

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 006/2016

Bauru, 31 de agosto de 2016.

Senhor Professor,

Informamos que a proposta intitulada ***Efeito protetor da Metformina no processo de remodelação óssea durante a aplicação de forças ortodônticas em ratos diabéticos tipo-2***, *registrada sob* ***CEEPA-Proc. Nº 006/2016,*** tendo Vossa Senhoria como Pesquisador Responsável, que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi analisada e considerada APROVADA a sua execução nas dependências da FOB-USP, em reunião ordinária da Comissão de Ética no Ensino e Pesquisa em Animais (CEEPA), realizada no dia 26 de agosto de 2016.

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização:	*Julho/2016 a Novembro/2017*
Espécie/linhagem/raça:	*Rato heterogênico/ Wistar albino*
Nº de animais:	*79*
Peso/Idade	*200g-250g/60 dias*
Sexo:	*Machos*
Origem:	*Biotério Central da PUSP/RP*

Esta CEEPA solicita que ao final da pesquisa 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,

Profª Drª Ana Paula Campanelli
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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