Effect of diabetes on orthodontic tooth movement in a mouse model


Orthodontic tooth movement is achieved by the remodeling of alveolar bone in response to mechanical loading. Type 1 diabetes results in bone remodeling, suggesting that this disease might affect orthodontic tooth movement. The present study investigated the effects of the diabetic state on orthodontic tooth movement. An orthodontic appliance was placed in normoglycemic (NG), streptozotocin-induced diabetes (DB), and insulin-treated DB (IT) C57BL6/J mice. Histomorphometric analysis and quantitative PCR of periodontium were performed. The DB mice exhibited greater orthodontic tooth movement and had a higher number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts than NG mice. This was associated with increased expression of factors involved in osteoclast activity and recruitment (Rankl, Csf1, Ccl2, Ccl5, and Tnfa) in DB mice. The expression of osteoblastic markers (Runx2, Ocn, Col1, and Alp) was decreased in DB mice. Reversal of the diabetic state by insulin treatment resulted in morphological findings similar to those of NG mice.

These results suggest that the diabetic state up-regulates osteoclast migration and activity and down-regulates osteoblast differentiation, resulting in greater orthodontic tooth movement.

Diabetes mellitus (Type 1 diabetes) is a metabolic disorder characterized by defects in either the secretion or the action of insulin, or both, leading to chronic hyperglycemia and disturbances of carbohydrate, fat, and protein metabolism (1). Diabetes may also affect bone turnover, resulting in diminished bone-mineral density, osteopenia, osteoporosis (2), and an increased prevalence and severity of periodontal disease (3–5). Several mechanisms have been reported to explain the altered bone remodeling in diabetes, one of which is diminished bone formation as a result of decreased osteoblastic activity (6–9) or enhanced apoptosis of osteoblastic cells (6, 10). Another contributing factor may be increased bone-resorptive activity (8, 10, 11). However, it is still controversial whether osteoclastic recruitment and function are altered in diabetes, because no change or decrease in the activity of osteoclasts has been reported (6, 7, 12).

Orthodontic tooth movement is achieved by the remodeling of alveolar bone in response to mechanical loading (13, 14). Bone resorption is caused by osteoclast activity in the compression side and by the osteoblast-induced formation of new bone in the tension side (13, 14). An alteration of the metabolic state that interferes with bone remodeling can result in a different rate of tooth movement (15). Therefore, diabetes may affect orthodontic tooth movement (16, 17).

The recruitment and activity of osteoclasts and osteoblasts are influenced by chemokines, cytokines, and bone-remodeling regulators (18–20). Recent reports demonstrated increased expression of mRNA for Ccl2, Ccl5, tumor necrosis factor-alpha (Tnfa), and receptor activator of nuclear factor-xB ligand (Rankl) that are associated with osteoclast recruitment and activity during orthodontic movement (21–24). Previous investigators have reported that diabetes is associated with prolonged expression of mRNA for Tnfa, Ccl2 (25, 26), Rankl, and colony-stimulating factor 1 (Csf1) (4, 27), which may lead to more persistent inflammation and tissue damage (25, 26). However, the cellular and molecular mechanisms associated with the diabetic state that may influence orthodontic movement are not known.

The aim of this study was to evaluate the effects of Type 1 diabetes in osteoclast recruitment and activity and, consequently, in orthodontic tooth movement in a mouse model (21).
Material and methods

Experimental animals

Sixty, 10-wk-old male C57BL6/J mice were used in this experiment. All animals were treated according to the ethical regulations for animal experiments, as defined by the Institutional Ethics Committee of Universidade Federal de Minas Gerais UFMG (135/08). Thirty-five, 7-wk-old mice (each weighing 20–25 g) were rendered diabetic (DB) with Type 1 diabetes by intraperitoneal injection of 120 mg kg\(^{-1}\) of streptozotocin (STZ; Sigma-Aldrich, St Louis, MO, USA), freshly dissolved in citrate buffer (0.1 mol l\(^{-1}\); pH 4.5; 28). The mice were fasted for 8 h prior to STZ injection. Seven days after induction, the plasma glucose levels of blood samples collected from the tail vein were determined using a glucose-oxidase enzymatic method (Accu-Check Advantage; Roche, Mannheim, Germany). Type 1 diabetes mellitus was confirmed by the presence of a blood glucose concentration of \(>300\) mg dl\(^{-1}\) after 8 h of fasting. The injection of STZ was repeated up to four times at intervals of 1 wk when glucose levels of \(<300\) mg dl\(^{-1}\) were detected. Normoglycemic (NG) mice received only citrate buffer solution (PBS). Ten of the DB mice received daily (morning and evening, respectively) intraperitoneal injections of Insulin N and Insulin R (Novo Nordisk, Bagsvaerd, Denmark). The doses were individually adjusted to maintain non-fasting blood glucose levels of 90–190 mg dl\(^{-1}\) over a 4 wk time-period. Controls were treated with vehicle (PBS). Weight and plasma glucose concentration were recorded during the experimental period. Blood samples collected when animals were killed were used to evaluate the concentration of glycated hemoglobin. Histopathological analysis of the pancreas was also performed.

 Experimental protocol

Induction of tooth movement was performed as previously described (21). Briefly, mice were anesthetized intraperitoneally with 0.2 ml of a solution containing xylazine (0.02 mg ml\(^{-1}\)) and ketamine (50 mg ml\(^{-1}\)). An orthodontic appliance consisting of a nickel-titanium (Ni-Ti) 0.25 × 0.76 mm coiled spring (Lancer Orthodontics, San Marcos, CA, USA) was bonded with a light-cured resin (Transbond, Unitek/3M, Monrovia, CA, USA) between the maxillary right first molar and the incisors (Fig. 1). The force magnitude was calibrated by use of a tension gauge (Shimpo Instruments, Itasca, IL, USA) to exert a force of \(35\) g applied in the mesial direction. There was no reaction during the experimental period. The animals were divided into three groups: DB mice, NG mice, and insulin-treated DB (IT) mice. For histomorphometric analyses, the left side of the maxilla was used as the control. For biochemical analyses there were two groups: the control group (comprising the non-operated animals) and the experimental group (comprising those fitted with an activated coiled spring). Mice were killed with an overdose of anesthetic at the following time-points: 6 and 12 d for histological measurements; and 0, 12, and 72 h for biochemical analyses. For every set of experiments, five animals were killed at each time-point.

Histopathological analyses

The right and the left halves of the maxillae, including the first, second, and third molars, were dissected, fixed in 10% buffered formalin (pH 7.4), and rinsed in distilled water. After fixation, each hemimaxilla was decalcified in 14% EDTA (pH 7.4) for 20 d and embedded in paraffin. The samples were cut into sagittal sections of 5 μm thickness. The selection of samples was based on morphological criteria, such as the position of the first molar disto-buccal root, where it appeared to be as long as possible. The sections were stained for tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich), counterstained with hematoxylin, and used for histological analyses. Five sections per animal of the first molar distal-buccal root, on its coronal two-thirds of the mesial periodontal site, were used for osteoclast counts, and the mean of those five sections was used as the result for that animal. Osteoclasts were identified as TRAP-positive, multinucleated cells sited on the bone surface. The total number of TRAP-positive cells was determined in five consecutive microscopic fields (\(\times 40\) magnification), comprising a total area of 0.1 mm\(^2\). The slides were counted by two examiners, and the intra-class correlation coefficient showed average measures of 0.985, validating the measurement.

Measurement of tooth movement

Image J software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the amount of tooth movement, as previously described (21). This was performed by measuring the distance between the cemento–enamel junctions (CEJs) of the first molar and the second molar (first and second molar distance), in five vertical sections per animal, under a microscope (Axioskop 40; Carl Zeiss, Göttingen, Germany) adapted to a digital camera (PowerShot A620; Canon, Tokyo, Japan). Three measurements were conducted for each evaluation and the variability was below 5% in all cases.

RNA extraction and real-time PCR

Using a stereomicroscope, periodontal ligament and surrounding alveolar bone samples were extracted from the
upper first molars. The gingiva, oral mucosa, and tooth were dissected and discarded, and RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using 2 μg of mRNA through a reverse transcription reaction (Superscript II; Invitrogen). Real-time PCR analysis was performed in an ABI Prism 7000 using a SYBR-green fluorescence quantification system (Applied Biosystems, Foster City, CA, USA). Standard PCR conditions were 95°C (10 min), and then 40 cycles of 94°C (1 min), 58°C (1 min), and 72°C (2 min), followed by the standard denaturation curve. Primer sequences are given in Table S1.

Statistical analysis

The mean cycle threshold (Ct) values from duplicate measurements were used to calculate expression of the target gene, with normalization to an internal control (β-actin), using the 2−ΔΔCt formula. Values obtained for the experimental groups were normalized to the respective controls. Thus, the average values of basal expression levels in control groups (without mechanical loading) were assigned as value of one and were not presented in the graphs.

Results

Blood glucose levels

The fasting blood glucose levels of NG mice were 145 ± 9.8 mg dl−1, whereas the fasting blood glucose levels of DB mice were 483 ± 24.4 mg dl−1. The hyperglycemic state was maintained during the entire experimental course. The glycated hemoglobin levels were significantly increased in DB mice (7.8 ± 1% Hba1c) relative to the levels in NG mice (4.3 ± 0.75% Hba1c; P < 0.05). Insulin treatment significantly reversed the diabetic state (blood glucose, 173 ± 30.5 mg dl−1; glycated hemoglobin, 4.1 ± 0.75% Hba1c; P < 0.05). Following treatment with streptozotocin, pancreatic islets of DB mice presented disorganization, size reduction, and loss of architecture in comparison with those of NG mice (data not shown). There were no significant weight changes between groups during the experiments (data not shown).

Tooth movement and TRAP-positive cells

The results demonstrated a greater amount of tooth movement in DB mice at 6 and 12 d of mechanical loading compared with NG mice and IT mice at the same time-points (Fig. 2A; P < 0.05). The number of TRAP-positive osteoclasts was increased at 6 and 12 d in all groups subjected to orthodontic force (Fig. 2B; P < 0.05). However, higher numbers of TRAP-positive osteoclasts were observed in DB mice (Figs 2B and 3D,F,H) than in NG mice (Figs 2B and 3C,E,G) and IT mice (Fig. 2B; P < 0.05). Overall, there was a good correlation between the intensity of TRAP activity, as evaluated qualitatively, and the number of TRAP-positive osteoclasts.

Cytokine and chemokine expression

There was an increase in the expression of mRNA for Ccl2, Ccl5, and Tnfa in DB and NG mice at 12 and 72 h of mechanical loading, and this increase was greater in DB mice than in NG mice (P < 0.05; Fig. 4A–C).

Osteoclastic and osteoblastic markers

Periodontal tissues of NG mice and DB mice showed a significant increase in the expression of mRNA for receptor activator of nuclear factor-κB (Rank), Rankl, matrix metalloproteinase-13 (Mmp13), and Csf1, 12 and 72 h after mechanical loading. Although there was no significant difference in the levels of mRNA for Mmp13 and Rank (Fig. 5A,B), the levels of mRNA for Rankl...
and Csf1 (Fig. 5C,D) were significantly higher in DB mice than in NG mice after 72 h of orthodontic force ($P < 0.05$).

The levels of mRNA for Alp, Col1, Runx2, and Ocn were significantly higher in both groups after 72 h of mechanical loading. Nevertheless, the levels of these markers were significantly lower in DB mice compared with NG mice at the same time-points ($P < 0.05$; Fig. 6A–D).

**Discussion**

Bone is a tissue that undergoes constant remodeling as a result of bone resorption and new bone formation (13, 14). These processes can be disturbed by diabetes (2, 6, 10, 12). Orthodontic tooth movement depends on balanced alveolar bone remodeling (13, 14); therefore, it can be hypothesized that diabetes might affect this process. However, little is known about how diabetes affects orthodontic tooth movement. Our results demonstrated that DB mice presented an enhanced number of TRAP-positive cells, increased bone resorption and, consequently, a greater amount of tooth movement. In accordance, higher levels of TRAP activity (8, 11) and an increased number of osteoclasts were observed in diabetic rats under orthodontic tooth movement (17) and other (27, 29) models. On the other hand, some previous studies indicated either no change, or a decrease, in the number of osteoclasts in diabetic rodents (6, 12). The reasons for these discrepancies are probably associated with the distinct models used, namely a model of infection to induce bone loss (6) and a model of physiological bone turnover, where no inflammation is present (12) (i.e. conditions that contrast with the bone remodelling induced by mechanical loading). The studies that applied experimental conditions similar to those of the present study, such as bone healing (27) and orthodontic tooth movement (17), also reported results similar to ours. However, in the study of Li et al. (17), data concerning
some morphometric and biochemical parameters were lacking.

The histopathological data revealed that insulin therapy resulted in the normalization of osteoclast numbers and of tooth movement in DB mice. In support of this, control of glucose blood levels with insulin prevented disturbance in bone turnover in other models (12, 30, 31).

Type 1 diabetes is also associated with an augmentation of cytokine production, leading to a persistent stimulus for leukocyte recruitment and for the maintenance or amplification of inflammation (25, 26). A significant increase in the expression of mRNA for Ccl2, Ccl5, and Tnfa was observed in periodontal tissues under mechanical loading in DB mice compared with NG mice. These molecules were shown to be increased in response to orthodontic loading in NG mice (21–24). Other studies also demonstrated an increased expression of mRNA for Ccl2 (25, 26, 32),
TNFα (3, 25–27, 33), and CCL5 (34) in DB mice. The higher levels of pro-inflammatory cytokines and chemokines might explain the enhanced number of osteoclasts and the more severe bone resorption in the diabetic group.

Markers of osteoclast activity and recruitment, such as Rank, Rankl, Mmp13, and Csf1 were increased in the periodontal tissue of both NG and DB mice after mechanical loading. The data also revealed a significant enhancement in the levels of mRNA for Rankl and Csf1 in DB mice. Previous studies have demonstrated elevated levels of Rankl (4, 27), Csf1 (27) and other osteoclast markers, such as Trap (8, 11), Mmp2, Mmp9 (33), and cathepsin K (8) during the diabetic state. The results supported the hypothesis that the up-regulation of expression of Rankl and Csf1 genes, associated with an increased number of osteoclasts, might result in increased bone resorption and greater tooth movement. These findings are also corroborated by studies that demonstrated increased osteoclastic activity during the early stages of Type 1 (8, 17, 27) and Type 2 (10, 11) diabetes, which contributed to the bone loss in diabetic rats and humans, respectively. Nevertheless, some studies reported no change, or a decrease, in osteoclastic activity in DB mice (6, 12). However, no significant differences were observed in the levels of mRNA for Rank and Mmp13 between NG mice and DB mice. In accordance, in a fracture model, the levels of mRNA for Mmp13 and Rank were similar between DB mice and NG mice on days 12 and 16 (27).

Previous studies have reported that the osteoblast/osteoclast interaction chiefly regulates bone remodeling (18–20). Our results demonstrated that Type 1 diabetes leads to a decreased expression of osteoblastic markers, such as Runx2, Alp, Ocn, and Coll. Other studies also demonstrated a decreased expression of Ocn (5–9), Alp (8), and Runx2 (9) during the diabetic state. Taken together, the results suggest that diminished differentiation of osteoblasts in DB mice might lead to reduced inhibitory signals for osteoclasts, resulting in increased alveolar bone resorption and greater tooth movement.

In conclusion, this study demonstrated that more tooth movement occurs in DB mice than in NG mice. This phenotype is reversed when hyperglycemia is controlled by insulin. Altogether, this study suggested that uncontrolled Type 1 diabetes alters alveolar bone turnover by uncoupled osteoblast/osteoclast function and augmented levels of pro-inflammatory mediators, leading to increased bone resorption and a greater amount of orthodontic tooth movement.

Acknowledgements – We are grateful to Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Fig. 6. Expression of mRNA for the osteoblastic markers Alp (A), Coll (B), Runx2 (C), and Ocn (D) in the periodontium of normoglycemic (NG) and diabetic (DB) mice after 12 and 72 h of mechanical loading. The boxes depict the real-time PCR melting curve for the respective gene targets. Values obtained for the experimental groups were normalized to the respective controls. The data are expressed as mean ± SEM. #P < 0.05 comparing NG with DB experimental groups. Data were evaluated using one-way ANOVA and the Newman–Keuls multiple comparison test.
References


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Supporting Information
Additional Supporting Information may be found in the online version of this article:

Table S1. PCR primer sequences and reaction properties.

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