Effect of Type 1 Diabetes on Orthodontic Tooth Movement in Extraction Cases: Study in Rat Model, As Examined by Histological and Real-Time Pcr Gene Expression Analysis

Maryam Sarbaz

Department of Orthodontics, Shiraz University of medical sciences, Shiraz, Iran

Corresponding Author*

Maryam Sarbaz
Department of orthodontics
Shiraz University of Medical Sciences
Shiraz
Iran
E-mail: sarbazmaryambe@gmail.com

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Abstract

Context: 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.

Aim: The present study investigated the effect of type 1 diabetes on tooth movement in extraction rat models.

Materials and Methods: Diabetes was induced by administering a single intraperitoneal injection of streptozotocin. Rats with a blood glucose level exceeding 300 mg/dl were assigned to the diabetes group. Insulin was administered daily to the diabetes+insulin group. An orthodontic appliance was placed in Normoglycemic (NG), streptozotocin-induced diabetes, and Insulin-Treated DB (IT) Sprague-Dawley rats. The first maxillary left molars were extracted. A nickel–titanium closed-coil spring of 50 g was applied for 2 weeks to the maxillary left second molar in all rats to induce mesial tooth movement. Histopathological analysis and quantitative PCR of periodontium were performed.

Results: The NG rats exhibited greater orthodontic tooth movement and had a higher number of multinucleated osteoclasts than diabetic rats. This was associated with decreased expression of factors involved in osteoclast activity and recruitment (Rankl) in NG rats. Reversal of the diabetic state by insulin treatment resulted in findings similar to those of NG rats.

Conclusion: In rats, diabetes caused the loss of effective orthodontic tooth movement. The regulation of blood glucose level by insulin administration largely reduced these abnormal responses on orthodontic force.

Introduction

The response of tissue to orthodontic force can be affected by various local and systemic disorders (1). Among these disorders, diabetes mellitus is an endocrine disorder affecting a large number of populations worldwide. In 2017, it was estimated that there are approximately 451 million diabetic individuals in the world, and that number is expected to reach 693 million by 2045 (2).

Type 1 diabetes (T1D), caused by an autoimmune destruction of beta-cells in the pancreas with resulting insulin deficiency, is a chronic condition often diagnosed in childhood or early adult life (3,4). Long-standing T1D is associated with a variety of complications such as delayed wound healing, stroke, renal failure, anxiety, retinopathy, and limb amputation (5,6). Most of these complications are the result of capillary damage observed in the diseased.

Diabetes may also affect bone turnover (7,8) resulting in diminished bone mineral density (9), osteopenia, osteoporosis (10-13) and an increased prevalence and severity of periodontal disease (14-15).

One of the primary effects of diabetes is increased inflammation in various tissues (16). Recent studies show that the application of orthodontic force in diabetic rats produces more inflammatory response than in (NG) rats. (17)

Orthodontic tooth movement depends on balanced alveolar bone remodeling; therefore, it can be hypothesized that diabetes might affect this process. 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 or enhanced apoptosis of osteoblastic cells (18-20). In addition, clinical studies have also shown that diabetes induces an increase in the production of pro-inflammatory factors which accelerate bone resorption (21).

Another contributing factor may be increased bone resorptive activity. 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 (22).

To date, three studies have investigated the effect of DM on the rate of orthodontic tooth movement. In the study by Braga et al., accelerated orthodontic tooth movement was detected in diabetic mice. A higher osteoclast count suggested that DM induces orthodontic tooth movement by induced proliferation of osteoclasts.in addition (23), in recent study by Vicente, showed that Mechanical stress in untreated-diabetic rats produces more inflammatory response, tooth movement, and PDL disorganization than among NG rats (24). However, observations by Arita et al., indicated that DM reduces resorption of the bone leading to a reduced rate of orthodontic tooth movement. However, this study did not observe the effect of DM on bone cells or periodontal biomarkers; so, the exact reason for the diminished orthodontic tooth movement was unclear (25). So, it is still controversial whether tooth movement increased in diabetic patients or decreased.

All studies that examined the effect of diabetes mellitus on tooth movement were performed in non-extraction scenarios and atraumatic conditions, while Extraction is a procedure that is performed routinely in orthodontics, either in the context of early treatment (serial extraction), for adolescents with severe crowding or protrusion/overjet, or for adult patients who have fewer possibilities for expanding the dental arch (26).

Delayed and non-healing of soft and hard tissues is common in diabetic patients (27,28). One prospective cohort study has examined in healing rates exist, following tooth extraction in type 1 insulin dependent diabetics, as compared to a control group. This study concluded that a higher incidence of delayed healing in insulin dependent patients which supports the medical evidence11 of a higher risk of infection and delayed healing for insulin dependent diabetic patients (29).

The mechanisms involved in orthodontic tooth movement are similar to those observed during a normal healing process, because diabetes alter

the normal healing process in diabetic cases, so the purpose of this study will be to investigate the effect of type 1 diabetes on tooth movement in extraction rat models.

Materials and Methods

Experimental animals

A total of 48 adult male Sprague-Dawley rats, weighing between 220 and 250 g (mean weight 231 g), were included in this experimental study, which took place between January 2019 and October 2020. The study was approved by the Ethical Committee Board and the experimental procedures were performed in accord with the ARRIVE guideline (Animal Research: Reporting of *in vivo* Experiments, Available at: www.nc3rs.org.uk/ARRIVE.17).The rats were housed in individual ventilated cages with 12/12 light/dark cycles and fed and watered ad libitum. The animals were divided into three groups (n=16 per group): group A Normoglycemics (NG), group B diabetics without treatment, and group C insulin treated diabetics. An identical orthodontic force applied for all groups.

Induction of type 1 diabetes mellitus

Type I diabetes was induced on day 1 of the experiment in groups B and C by administering a single intraperitoneal injection of Streptozotocin (STZ) (Sigma-Aldrich Chemistry, S.A., Madrid, Spain), 65 mg/kg body weight dissolved in freshly prepared citrate buffer pH 4.5 (30). Fasting blood glucose was evaluated prior to the injection of STZ and After 72 hr of intraperitoneal administration of STZ. Blood glucose levels were monitored puncturing each rat's tail vein and measuring glucose levels with an auto coding blood glucose meter EasyGluco™ (Osang Healthcare South Korea, infopia, KTM). Animals presenting blood glucose levels over 300 mg/dl were considered diabetic. Blood glucose levels were checked regularly and continued to present type 1 diabetes throughout the study (31).

Insulin treatment

Insulin administration began in group C animals 3 days after STZ injection (once glucose levels>300 mg/dl had been confirmed). They were injected daily with human insulin subcutaneously (lansulin N, Exir pharmaceutical, Iran), adjusting the dose to the requirements of each animal (32). Groups A and B were injected with saline solution. Because hypoglycemia by insulin administration sometimes causes threat of life, glucose control level in the group was settled to be still higher than normal level (120 mg/dl) in the group; the same amount of saline (0.1 ml) was administered to the control and diabetic groups

Installation of the orthodontic appliance and tooth extraction

On day 7 of the experiment, orthodontic force application and tooth extraction were performed under general anesthesia by intra-muscular injection of ketamine hydrochloride (Brermer pharma GMBH, Germany) at a dose of 90 mg/kg in combination with xylazine hydrochloride (Alfasan, Woerden, Holland) at a dose of 10 mg/kg. When general anesthesia was achieved, the first maxillary left molars (M1) were extracted in each rat with dental explorer (Figure 1a). The tip of this instrument was being first placed at the disto-buccal gingival margin between the first and second molars. The dental explorer repeatedly rotated in a dorsal and mesial direction to loosen the first molar (33(. After extraction, mechanical stress was loaded in all groups by inserting a standardized nickle-titanium close coil spring (American Orthodontics NiTi closed coil, 010 x 030 inch, 9mm/Eyelet) set between upper left second molars and the incisors (Figure 1b). The force level of the coil spring after activation was approximately 0.5N in keeping with previous reports indicating that application of 0.5N does not damage periodontal tissues (30).

The springs were not reactivating during the course of the study. The coil spring fixed with a 0/010-inch stainless steel ligature wire (3M Unitek, Monrovia, CA, USA). Light-cure flowable composite resin (DenFil Flow, Vericom Co., Korea) used to bind the upper incisors, therefor limiting their dislocation and enforcing anterior anchorage.

No orthodontic force was applied to the upper right second molar (contralateral control side) in either group. In order to minimize animal discomfort and protect their appliance, lower incisor bind with light-cure flowable composite resin and standard chow finely ground and moistened with tap water. 8 rats in each group were sacrificed under general anesthesia overdose at 2week after orthodontic appliance placement to evaluate the osteoclast number and real-time PCR.

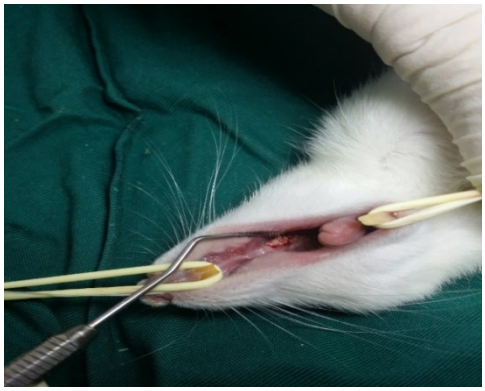

Figure 1a. First molar extraction.

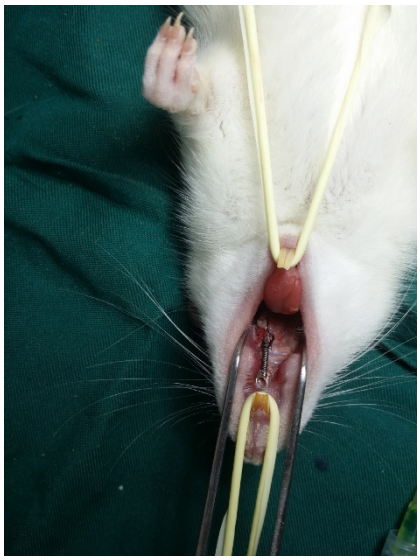

Figure 1b. Occlusal view of a nickel-titanium (Ni-Ti) open coil spring placed between the upper left second molar and the incisors.

Measurement of tooth movement

At the experimental end-points, all rats weighed by digital scale (Shimadzu, Kyoto, Japan, 61189) and scarified by means of drug overdose. Maxilla separated and the distance between the distal surface of the second molar and the mesial surface of third molar measured by a digital caliper (Mitutoyo Co., Kawasaki-Shi, Japan) before appliance removal. To reduce the measurement errors, each space measured by three persons separately, and the mean value used as the final measurement.

Histological Analysis

Tissue preparation

After tooth movement measuring at the end of the experiment, half maxillas in each group including the first molar socket, second and third molar dissected and fixed in a 10% buffered formalin solution (pH 7.4). The fixed maxillas were washed in distilled water for 5 min and then immersed in a decalcification solution. The samples were cut into sagittal

sections of 5 lm thickness. For this purpose, 5 sections containing the largest root area and including the entire length of the molar root were selected and osteoclast number was assessed under an Olympus BX51 light microscope.

RNA extraction and real-time PCR

Periodontal ligament and surrounding alveolar bone samples were extracted from the upper left second molars. The gingiva, oral mucosa, and tooth were dissected and discarded, and RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA).

A small cube of periodontal ligament and surrounding alveolar bone was homogenized to powder in liquid nitrogen and total RNA extraction was performed by total RNA extraction kit (pars Tous, IRAN) according to the manufacturer's protocol. RNA samples were checked regarding quality and quantity using a spectrophotometer. Samples with ratios of absorbance at 260 nm and 280 nm ranging from 1.8 to 2.0 were selected. Also, the integrity of the RNA preparations was examined by agarose gel electrophoresis.

Complementary DNA (cDNA) was synthesized using 1000 ng of DNase treated RNA through a reverse transcription reaction) Applied Biosystems, USA) the cDNA synthesis process was done according to the manufacturer's instructions. Quantitative real-time PCR (QT-PCR) was performed using a Step One Real-time PCR thermocycler (Applied Biosystems, USA), SYBR green master mix (Amplicon, Denmark), and specific primers. Primers used for OPG, RANK, RANKL, and GAPDH are listed in Table1. PCR was run as 40 cycles at 95 °C for 15s, Annealing Temperature for 30s, and 72 °C for 30s.

The expression levels of OPG, RANK and RANKL were normalized to GAPDH mRNA levels. Data of target mRNA copies were calculated relative to GAPDH using the 2–ΔCt method (Table 1)

Gene name	Primer sequence	Product size	Annealing Temperature
RANK	Forward : 5'ACGGAATCAGATGTGGTC 3'	80	50˚c
	Reverse :5' AGACTGGGCAAGTAAACC3'		
RANK L	forward :CCGTGCAAAGGGAATTA CAA	139	54˚c
	reverse: GCATTGATGGTGAGGTGAGC		
OPG	Forward 5′-ACAATGAACAAGTGGCTGTGCTG-3′	109	58.5˚c
	Reverse 5′-CGGTTTCTGGGTCATAATGCAAG-3′		
GAPDH	forward: 5′-GATCGTGGAAGGGCTAATGA-3′	154	54˚c
	reverse: 5′-GACTTTGCCTACAGCCTTGG-3′		

Table 1. Quantitative RT-PCR primer sets.

Statistical analysis

The data of each group were expressed as mean ± SEM. Comparison among the groups was analyzed statistically using one-way Analysis Of Variance (ANOVA) followed by the Tukey test ($p < 0.05$).

Results

By the end of the experiment, one rat had died in the IT group (during the tooth-movement period). Appliance failure was noticed in two rats in the diabetic group.

The final size of the control, diabetic, insulin treated groups was 16, 14, and 15 rats, respectively.

Blood glucose levels

Blood glucose levels of NG rats were 127 ± 15.5 mg/dl, whereas the blood glucose levels of DB rats were 427 ± 61.42 mg/dl. The hyperglycemic state was maintained during the entire experimental course. Insulin treatment significantly reversed the diabetic state (blood glucose, 149 ± 25.9 mg/dl). The normal blood glucose level of the insulin administration group was maintained during the experimental course.

Because of the wounds caused by tooth extraction, the percentage weight loss occurred in all groups during the first week of experimental study. The results also revealed that, in the untreated diabetic rats, the mean body weights were reduced more in comparison with two other groups but it was not significant.

Orthodontic tooth movement

The results demonstrated a greater amount of tooth movement in NG rats at 2 week of mechanical loading compared with diabetic rats at the same time-points (Figure 2; $P<0.05$). Significant differences were not identified between NG and insulin-treated rats ($p \geq 0.05$).

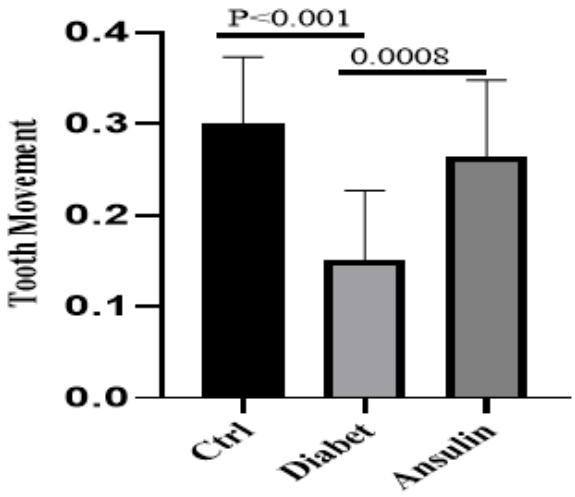

Figure 2. Amount of tooth movement in each group.

Histological analysis

Osteoclast numbers were significantly more in the NG group than untreated diabetic group. There was no significance difference in this variable among the NG group and insulin treated group (Figure 3).

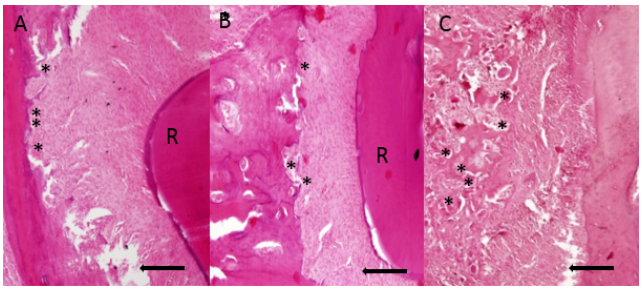

Figure 3. Histological changes related to orthodontic tooth movement. Sagittal sections are shown of the periodontium around the mesio-buccal root of the second molar in (NG) (A), diabetic (B) and insulin (c) groups. The large arrows indicate the direction of tooth movement. DB-distal alveolar bone; R-root. Osteoclasts are indicated by asterisks (Hematoxylin and Eosin staining; Original magnification × 200; scale bar for both images: 50 μm).

Osteoclastic Markers

The levels of mRNA for Rankl were significantly higher in NG and treated diabetic rats than in untreated diabetic rats after 2weeks of orthodontic force ($P < 0.05$). Although there was no significant difference in the levels of mRNA for Rankl in NG and treated diabetic rats (Figure 4).

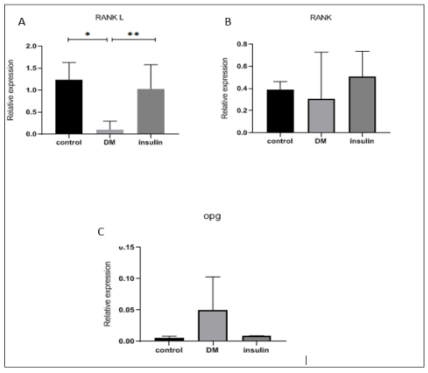

Figure 4. Gene expression levels of A, RANKL; B, RANK; and C, OPG in the periodontium of Normoglycemic (NG) and Diabetic (DB) rats after 2 weeks of mechanical loading. The data are expressed as mean ± SEM. #P < 0.05, NG vs. DB experimental groups. Data were evaluated using one-way ANOVA followed by post-hoc test.

Chapter Five: Discussion and Conclusion

Diabetes not only causes acute, potentially lifethreatening ketoacidosis (predominantly in patients with type 1 diabetes), but also damages many organs and tissues. The major long-term complications of the disease are related to blood vessel damage. Diabetes doubles the risk of cardiovascular disease (34), and approximately 75% of deaths in diabetics are due to coronary artery disease (35). From these facts, diabetes is recognized as a disease primarily characterized by vascular lesions. In this study, the effects of diabetes on orthodontic tooth movement were studied in extraction situations. The present study was performed in rats. Although there are some morphological and physiological differences between rat and human alveolar bone and periodontal ligament, rats are considered to be a good model to study orthodontic tooth movement and have been used extensively in previous studies (36). To induce diabetes in rats, STZ injection was used. STZ is a selective toxic agent targeting pancreatic β cells, causing irreversible diabetes. As the resultant diabetes is due to the destruction of pancreatic cells, it is similar to insulin dependent type I diabetes mellitus in humans (37).

In order to induce tooth movement in the present study, the force system that was used to induce tooth movement, was similar to that which was used by Li et al. in their study (30). However, in order to reduce the stress produced in the animals, we did not perform a shallow groove to fix the ligature to the incisors; we used a composite adhesive to boost retention.

Bone is a tissue that undergoes constant remodeling as a result of bone resorption and new bone formation. These processes can be disturbed by diabetes. Orthodontic tooth movement depends on balanced alveolar bone remodeling; therefore, it can be hypothesized that diabetes might affect this process. However, little is known about how diabetes affects orthodontic tooth movement (22). Our results demonstrated that NG rats presented an enhanced number of osteoclast, increased bone resorption and, consequently, a greater amount of tooth movement. However, conflicting results have been reported regarding the influence of diabetes on the amount of orthodontic tooth movement. Braga et al. (22) and Vicent et al. (16) reported that orthodontic tooth movement was increased under diabetes. Both studies were performed in atraumatic conditions without any tooth extraction. Extraction causes inflammatory histopathologic changes around the tooth that might affect tooth movement. It has been proven that healing of tooth extraction sockets in poorly controlled diabetic patients is often delayed and accompanied by severe infection. In diabetes mellitus, abnormalities have been found in collagen metabolism, the rate of endothelialization, capillary basement membrane thickening, and in the amount of granulation tissue (29).

The other reasons for these opposite result are probably associated with the distinct models used, type of orthodontic appliance, force level applied, duration of force, etc. for instance Braga et al. (30) used mice as an animal model and applying 35 g of orthodontic force. Thirty-five g for an upper first molar in mice could correspond to more than one kilogram for a human upper first molar. Such a super heavy force may have given an orthopedic force on alveolar bone that was weakened by osteoporosis from diabetes.

Arita et al. reported that orthodontic tooth movement was decreased under diabetes, which was similar to the result in the present study. Observations by Arita et al. indicated that DM reduces resorption of the bone leading to a reduced rate of orthodontic tooth movement. However, their study did not observe the effect of DM on bone cells or periodontal biomarkers (24). The results of the present study can therefore be used to justify the outcome observed.

The RANK/RANKL/OPG system is important in determining osteoclast differentiation. We found decreased expression of the pro-osteoclastic factor receptor activator of nuclear factor κB ligand (RANKL) suggests that DM reduce bone resorption by primarily decreasing the proliferation of osteoclasts. The results supported the

hypothesis that the down-regulation of expression of Rankl, associated with a decreased number of osteoclasts, might result in decreased bone resorption and lower orthodontic tooth movement. These findings are also corroborated by Plut et al., (38) but they did not detect any differences in tooth movement between NG and diabetics. This difference in the amount of tooth movement can be due to difference in the type of diabetes mellitus. In accordance with the previous studies, T1DM and T2DM have distinct pathophysiological mechanisms, which may differently affect bone metabolism.

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

In this study, insulin administration in the diabetes + insulin group was performed every day to maintain the blood glucose level below 180 mg/dl. However, the administration of insulin to diabetic rats returned the blood glucose level to the average of 149 ± 25.9 mg/dl through the experimental period. This amount is still high compared with that of control groups (127 ± 15.5 mg/dl). This higher blood glucose level in the diabetes + insulin group may have caused the smaller amount of tooth movement in the control group; however this result was not statistically significant.

In conclusion, this study demonstrated that diabetes caused diminished orthodontic tooth movement. Furthermore, the results suggested that uncontrolled Type 1 diabetes alters alveolar bone turnover by osteoblast/osteoclast function and augmented levels of pro-inflammatory mediators, leading to decreased bone resorption and a lower amount of orthodontic tooth movement especially when teeth are extracted in uncontrolled situation. The regulation of blood glucose level by insulin administration largely reduced these abnormal responses on orthodontic force.

Conclusion

In conclusion, this study demonstrated that diabetes caused the loss of effective orthodontic tooth movement in extraction cases. This study suggested that uncontrolled Type 1 diabetes alters alveolar bone turnover by osteoblast/osteoclast function and augmented levels of pro-inflammatory mediators, leading to decreased bone resorption and a lower amount of orthodontic tooth movement. The regulation of blood glucose level by insulin administration was successful in reversing this phenomenon.

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