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# Alterations of collagen-I, MMP-1 and TIMP-1 in the periodontal ligament of diabetic rats under mechanical stress

**L. Zhang, X. Li, L.-J. Bi**
Department of Stomatology, The Fourth Affiliated Hospital, Harbin Medical University, Harbin, China

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*Background and Objective:* The present study assessed effects of hyperglycemia on production of proteins involved in remodeling of the periodontal ligament under mechanical stress.

*Material and Methods:* Forty-eight Sprague–Dawley rats were randomly divided into the following two groups: nondiabetic (ND) and diabetes induced (DI; $n = 24$ each group). Diabetes was induced in the DI group by a single dose of streptozotocin, and saline solution was injected in the ND group. Rats were killed 1–14 d after induction of mechanical pressure (50 g) on the first upper left molar. Alterations of collagen type I (Col-I), MMP-1 and TIMP-1 in the upper left periodontal ligament of these rats were measured immunohistochemically and compared with those on the contralateral side of the same rat (control; no force induction).

*Results:* The DI group showed a decrease in Col-I and an increase in MMP-1 compared with the ND group. Both Col-I and MMP-1 increased in both groups, whereas TIMP-1 was decreased following mechanical pressure. The DI group exhibited a longer duration of increased MMP-1 and MMP/TIMP ratio compared with the ND group.

*Conclusion:* Diabetes affects proteins involved in remodeling of periodontal ligament during mechanical pressure. This may delay the reconstruction and remodeling of periodontal ligament in diabetic individuals.

Liang-Jia Bi, DDS, PhD, Department of Stomatology, No. 37 Yinhang Street, Nangang District, The Fourth Affiliated Hospital, Harbin Medical University, Harbin 150001, China
Tel: +86 0451 82576565
Fax: +86 0451 82576502
e-mail: biliangjia66@163.com

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Diabetes mellitus is a systemic metabolic disorder characterized by impaired ability to utilize glucose and regulate blood glucose levels. This increasingly common endocrine disorder is associated with long-term microvascular complications as well as disturbances in collagen metabolism and delayed wound healing (1,2). Most evidence demonstrates an increase in the prevalence and severity of periodontal disease in patients with poorly controlled diabetes mellitus (3–6), reflecting the important association between diabetes and the pathogenesis of periodontal disease (7–9).

A number of epidemiological and other investigations have described the bidirectional inter-relationship exhibited by diabetes and periodontal disease (10–14). Several underlying mechanisms are likely to account for the association of diabetes with periodontal disease. Vascular changes caused by hyperglycemia are associated with the development of species of periodontal pathogens (15). Diabetic

patients also show an exacerbated host response, with hyperproduction of inflammatory mediators and polymorphonuclear leukocyte dysfunction. In addition, several oral diabetes-induced collagen abnormalities have been identified, including a large reduction in collagen synthesis and solubility in gingiva. These findings suggest that the disease increases the degradation of newly synthesized collagen in periodontal tissues.

Orthodontic treatment can be complicated in patients with diabetes, especially those with uncontrolled or poorly controlled disease, due to the presence of compromised periodontium (16,17). Collagen, especially collagen type I (Col-I), is the main structural component in the periodontal ligament and plays an important role during wound-healing processes and remodeling (18). Orthodontic tooth movement-associated periodontal remodeling can be partly attributed to the modulation of fibroblast gene production of proteases, such as MMP-1 (19). The balance of MMPs and TIMPs is central to the stabilization of the extracellular matrix, with an MMP–TIMP imbalance associated with pathological breakdown of the extracellular matrix in periodontitis (20–22). Such an imbalance may also reflect a disordered microenvironment due to uncontrolled hyperglycemia (23). This provides a biologically plausible basis for disturbed connective tissue modeling under orthodontic force in the presence of hyperglycemia.

Most knowledge regarding connective tissue modeling (including collagen, MMP and TIMP levels) has been derived from *in vitro* studies using cell lines or primary cultures (24–27). Studies in animal models are relatively few, and the effect of mechanical stress on Col-I, MMP-1 and TIMP-1 in the periodontal ligament in rodent models of experimental diabetes is still unclear. How hyperglycemia affects remodeling and reconstruction of the periodontal ligament is not fully elucidated. To better understand the pathological changes after mechanical stress in diabetic periodontal ligament, the present study explored the production and variation of Col-I, MMP-1 and TIMP-1 in the periodontal ligament of nondiabetic and streptozotocin (STZ)-diabetic rats with and without the application of orthodontic force.

## Material and methods

### Study animals

Forty-eight 6- to 7-wk-old male Sprague–Dawley rats (185–211 g) were randomly divided into the following two experimental groups: nondiabetic (ND) and diabetes induced (DI; $n = 24$ each group). Diabetes was induced in the DI group by a single intraperitoneal injection of 65 mg/kg streptozotocin (Sigma Chemical Co., St Louis, MO, USA). Saline solution was injected in the ND group. Fasting blood glucose was evaluated at regular intervals using the Accu-Chek Active monitoring system (Roche Diagnostics GmbH, Mannheim, Germany). Blood glucose was determined before orthodontic treatment and killing of study animals. Streptozotocin-injected rats were considered diabetic if fasting glucose levels were $>300$ mg/dL. Diabetes-induced and nondiabetic rats were treated identically except for injection of STZ or saline, respectively. After injection, all animals were housed in singly metabolic cages and fed standard rat chow and water *ad libitum*.

### Orthodontic induction

Eight weeks after STZ or saline solution injections, orthodontic force was loaded on the upper left first molar in both the DI and ND groups (induction side; Fig. 1) No orthodontic force was applied to the upper right first molar (contralateral control side) in either group. Mechanical stress was loaded in both DI and ND rats by inserting a standardized coil spring composed of nickel–titanium wire between the upper incisors and the left upper first molar (28). The orthodontic appliance was fixed with a 0.2 mm stainless-steel wire. To prevent detachment of the appliance from the surface of the incisor, a shallow groove was created approximately 0.5 mm from the gingiva of the upper incisors. The force level of the coil spring after activation was approximately 50 g. The nickel–titanium spring was removed immediately before killing the rats.

### Retrieval, processing and sectioning

Four rats in each group were killed at 1, 3, 5, 7, 10 and 14 d after mechanical induction. Rats were anesthetized (sodium pentobarbital, 40 mg/kg, i.p.) and perfused intracardially with 4% paraformaldehyde and then decapitated. The maxillary jaw was excised and divided into right and left segments. Soft tissues around the maxilla were removed except for the gingiva. To improve fixation, the maxillary jaw was postfixed in 4% paraformaldehyde for 24 h at 4°C. After fixation, specimens were decalcified for ≥4 wk in 15% EDTA at 4°C, washed, dehydrated and embedded in paraffin. The first upper molar from each rat was cut into 3-μm-thick sagittal sections. All procedures adhered to the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*.

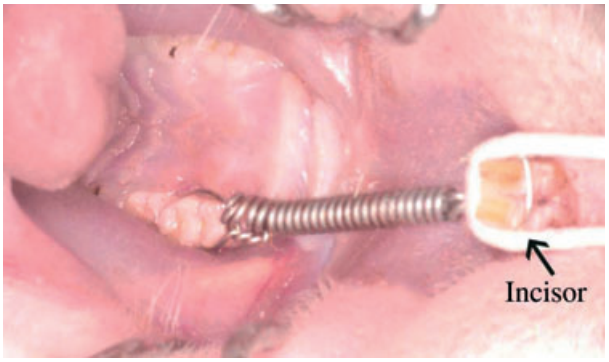



*Fig. 1.* Photograph of the orthodontic appliance placed to induce tooth movement in rats.

### Hematoxylin and eosin staining

Hematoxylin and eosin (H&E) staining and immunohistochemical analyses were carried out on the specimens. Slides were stained with H&E to identify changes in periodontal ligament fibers after mechanical induction and differences between the ND and DI groups. Color images were captured using a digital camera (D100; Nikon, Tokyo, Japan).

### Immunohistochemistry

Immunohistochemistry was conducted on deparaffinized 3-μm-thick sections. The sections were immersed in 3.0% $H_2O_2$–methanol for 20 min to block endogenous peroxidase activity. To recover antigen, sections were boiled in citrate buffer for 2 min and cooled naturally to room temperature. After rinsing three times in 0.01 M phosphate-buffered saline, the sections were incubated with blocking solution and then exposed to rabbit anti-Col-I antibody (1:180 dilution), rabbit anti-MMP-1 antibody (1:100 dilution) or rabbit anti-TIMP-1 antibody (1:80 dilution; Wuhan Boster Biological Technology Ltd, Wuhan, China) overnight at 4°C.

After rinsing with phosphate-buffered saline, the sites of bound primary antibody were detected by incubating sections with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1:600 dilution; Wuhan Boster Biological Technology Ltd) for 20 min. The sections were washed again and then immersed in a diaminobenzidine chromagen solution (0.5% diaminobenzidine and 0.03% $H_2O_2$) to visualize sites of antibody binding. The sections were developed in the diaminobenzidine solution until good color formation was observed by monitoring the reaction under a microscope. Finally, the sections were rinsed in distilled water, counterstained with Gill's II hematoxylin solution, dehydrated, and coverslipped with a xylene-based mounting medium.

Prior to immunostaining, tissue samples underwent an antigen retrieval procedure (29), which can potentially impair the quality of immunohistochemistry micrographs. Thus, MATLAB 7.0 software (Mathworks, Natick, MA, USA) was used to analyze the immunohistochemistry signal, with data presented graphically rather than in immunohistochemistry micrographs. The MATLAB 7.0 software was used to calculate the proportion of the periodontal ligament in area A is defined as apide side of periodontal ligament between the mesial side of the mesial root of the upper first molar and the mesial alveolar bone of the pressure side occupied by positive signals for Col-I, MMP-1 and TIMP-1 immunoreactivity.

### Statistical analysis

Data between ND and DI groups and between these groups and control were analyzed with Student's paired *t*-test and considered significant at $p < 0.05$. Data are expressed as means ± SD. Differences between time points within a group were determined by one-way analysis of variance, with statistical significance established at $p < 0.05$.

## Results

### Plasma glucose concentrations

Plasma glucose levels (in milligrams per deciliter) in the nondiabetic and diabetes-induced groups are shown in Table 1. The plasma glucose levels of the ND and DI groups did not differ at baseline. Following injection of STZ, plasma glucose level in the DI group (363.6 ± 46.9 mg/dL) was significantly higher than in the ND group (119.7 ± 37.8 mg/dL, $p < 0.001$). The plasma glucose level in the diabetes-induced group was higher than in the nondiabetic group before mechanical induction and at all time points thereafter (days 1, 3, 5, 7, 10 and 14, $p < 0.001$; Table 1). During mechanical induction, mean plasma glucose levels in both the ND and DI groups (104.2 ± 40.7 and 381.4 ± 69.0 mg/dL, respectively) did not change (101.6 ± 26.0 and 393.4 ± 22.2 mg/dL, respectively, $p > 0.05$).

### Histological findings

Figures 2 and 3 illustrate sections of periodontal ligament stained with H&E in nondiabetic and diabetes-induced rats. In the ND group, collagen was oriented normally and capillaries were normally distributed on the control side on day 0. On the induction side, the periodontal ligament in the ND group showed more irregular collagen and collapsed capillaries from day 1 to 7, but appeared normal on days 10 and 14. In the DI group, collagen was oriented irregularly and capillaries were distended on both the control side and the induction side at all time points. At the end of the experiment (day 14), the collagen fibers remained abnormally oriented.

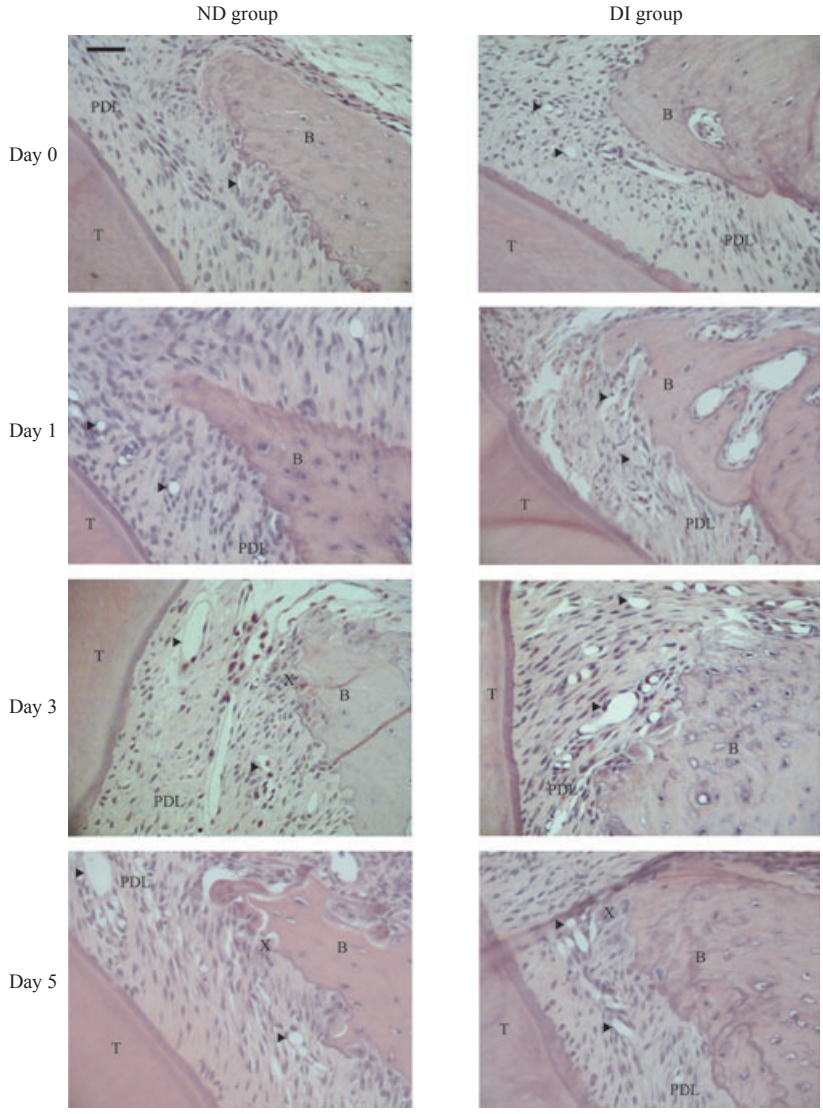
### Collagen-I, MMP-1 and TIMP-1 immunoreactivity in the periodontal ligament

Collagen-I, MMP-1 and TIMP-1 immunoreactivity were detected in the periodontal ligament of the ND and DI groups, and the between-group levels were significantly different following orthodontic induction (all $p < 0.05$;

*Table 1.* The blood glucose of the rats (mg/mL)

| | At study beginning | Post-STZ injection | Pre-orthodontic induction | Time after orthodontic induction and before killing (days) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 3 | 5 | 7 | 10 | 14 |
| ND group | 106.1 ± 21.1 | 119.7 ± 37.8 | 104.2 ± 40.7 | 98.3 ± 15.2 | 103.9 ± 19.2 | 113.3 ± 25.7 | 114.7 ± 13.1 | 97.1 ± 24.4 | 101.6 ± 26.0 |
| DI group | 112.9 ± 23.6 | 363.6 ± 46.9* | 381.4 ± 69.0* | 331.1 ± 19.6* | 361.2 ± 44.3* | 371.1 ± 11.8* | 362.3 ± 17.0* | 395.0 ± 21.0* | 393.4 ± 22.2* |

Abbreviations: DI, diabetes induced; ND, nondiabetic; STZ, strepozotocin. *$p < 0.05$ compared with the ND group.




T = tooth; B = alveolar bone; PDL = periodontal ligament; ► = capillary; X = osteoclasts; Day 0 = control side

*Fig. 2.* Photomicrographs on days 0–5 of sagittal sections of area A in the periodontal ligament of nondiabetic (ND) and diabetes-induced (DI) rats following mechanical induction. Sections were stained with hematoxylin and eosin. Arrowheads indicate capillaries. Abbreviations: B, bone; PDL, periodontal ligament; T, tooth; and X, osteoclast. Scale bar represents 200 μm.

Fig. 4). The DI group had a lower level of Col-I on the control side and induction side. Collagen-I in the ND group increased gradually after reaching a nadir on day 3 and approximated the level on the control side on days 10 and 14 (Fig. 4A); however, Col-I levels in diabetes-induced rats remained at lower levels until the end of study ($p < 0.05$).

The DI group had a significantly higher level of MMP-1 immunoreactivity on the control side than the ND group ($p < 0.05$). On the induction side, there was a transient increase in MMP-1 immunoreactivity in the ND group on days 1–3 followed by a gradual return to control levels on day 14. (Fig. 4B). The MMP-1 levels in the DI group remained stable for the first 3 d of the study and were significantly lower than in the ND group ($p = 0.05$). After day 7, MMP-1 levels increased markedly and remained elevated until day 14 ($p = 0.05$). TIMP-1 changed little in both groups on day 3 after orthodontic induction, but increased markedly on day 7 in the ND group ($p = 0.05$; Fig. 4C). There was slight but not significant change in the TIMP-1 level in the DI group during the entire study period. Levels of TIMP-1 were significantly different between the ND and DI groups from day 5 to 14 ($p < 0.05$), reflecting the increase in the ND group.

The ratio of MMP-1/TIMP-1 increased more in the DI group than in the ND group, with no significant difference on the control side ($p > 0.05$; Fig. 4D). After orthodontic induction, the ratio in the ND group increased rapidly until day 3 and then returned to the pre-induction level. In the DI group, the ratio did not change markedly until day 10, after which it remained elevated level until day 14 ($p > 0.05$). On day 14, the ratio of MMP-1/TIMP-1 was much higher in the DI group than in the ND group on the induction side ($p < 0.05$). Representative Col-I immu nohistochemical pictures of contralateral control sides and orthodontic induction sides in ND and DI rats on day 14 are shown in Fig. 5.

## Discussion

A positive relationship between hyperglycemia and immune response, inflammation and extracellular matrix synthesis has been definitively established (30–32). In addition, high glucose levels have been shown to inhibit the proliferation and differentiation of periodontal ligament fibroblast cells, which might partly account for delayed periodontal regeneration and healing in patients with diabetes (33). Alteration of Col-I occurs during reconstruction of the periodontal tissues, especially during periodontal wound healing (34). The remodeling of the periodontal ligament, which is mainly composed of collagens, is interfered with by disturbance in collagen metabolism in diabetes mellitus patients (7). In the present study, this process was more evident under orthodontic force. It is possible that hyerglycemia might account for the decreased production of Col-I seen in our data.

Compared with nondiabetic rats, diabetes-induced rats needed more

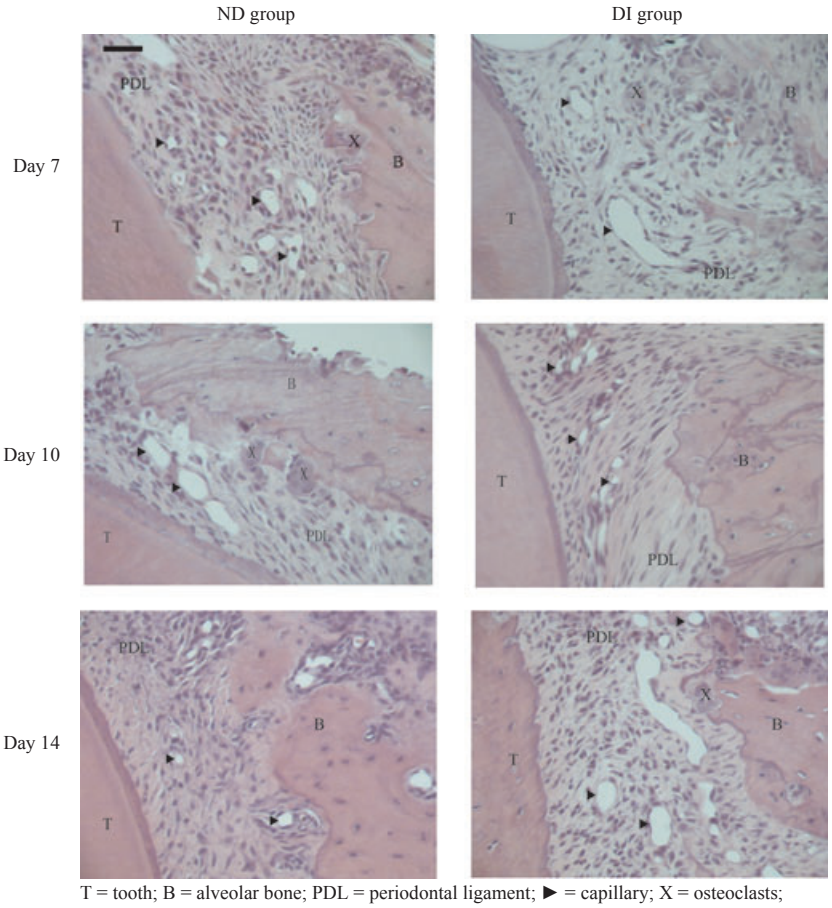



*Fig. 3*. Photomicrographs on Days 7–14 of sagittal sections of area A in the periodontal ligament of nondiabetic and diabetes-induced rats following mechanical induction. Sections were stained with hematoxylin and eosin. Arrowheads indicate capillaries. Abbreviations: B, bone; PDL, periodontal ligament; T, tooth; and X, osteoclast. Scale bar represents 200 μm.

time to complete changes in Col-I levels involved in periodontal ligament remodeling and reconstruction. Previous studies have documented this phenomenon in the remodeling of the periodontium due to occlusal force (35) and in diabetic mouse skin due to structural alterations in the intracellular matrix in hyperglycemic tissues (36).

Both MMP-1 and TIMP-1 affect the production of Col-I in the periodontal ligament directly or indirectly and play important roles in remodeling of the periodontal ligament during mechanical stress. However, MMPs have also been implicated in pathological diabetic conditions (e.g. cardiomyopathy) in animal models (37). Production of MMP-1, which originates from various inflammatory cells that invade the injured area, is increased in human periodontal ligament during mechanical force (19) and in dental tissues in patients with periodontitis (38). The MMP-1 causes degradation of collagen fibers and other extracellular matrix components. Evidence indicates that MMP-1 and periodontitis are closely correlated (39,40). Although TIMPs counter the effects of MMPs, an imbalance of MMPs over TIMPs can contribute to tissue destruction.

Recent findings underscore the potential role of phagocytosis incorporating denatured collagen in promoting more active remodeling of new extracellular matrices and ultimately tissue regeneration (41). At the mechanistic level, evidence supports the mechanistic contribution of mechanotransducing molecules on executioners of extracellular matrix remodeling in periodontal ligament cells undergoing mechanotransduction (42). In particular, strain-dependent mechano/signal transduction in periodontal ligament cells involves abundance and activity of focal adhesion kinase, MAP-kinases p42/44 and p38 stress kinase in conjunction with changes in levels of MMPs.

Our results indicated that the production of MMP-1 increases soon after orthodontic force is applied to the periodontal ligament and catabolizes Col-I, while TIMP-1 increases in the later stages to stop further damage to Col-I. In the ND group, the ratio of MMP-1/TIMP-1 increased only in the early stages of the study and then stabilized at control side levels, suggesting that the destruction of collagen in nondiabetic periodontal ligament is only transient and is rapidly arrested. However, in the diabetes-induced group, the imbalance of MMP-1 and TIMP-1, which emerged on day 5, persisted until the end of the study. This may be due to the robust production of MMP-1 from day 5 onwards and 'nonreaction' of TIMP-1. The higher ratio of MMP-1/TIMP-1 in the diabetes-induced group may lead to greater and more sustained destruction of Col-I in the periodontal ligament than in the diabetes-induced group.

The effect of hyperglycemia on the production of MMP-1 and TIMP-1 following orthodontic induction suggests an imbalance between the degradation and the synthesis of extracellular matrix in diabetes-affected tissues. This process may be responsible for increased tissue breakdown seen in diabetes. In addition, it has been shown that diabetic rats have increased inflammation and a more persistent inflammatory response following injury (30).

Previous studies suggest that periodontal disease affects the metabolic state in diabetes mellitus, although the evidence is not unequivocal (43). Holtzhausen *et al.* (43) found significantly higher glucose levels in streptozotocin-diabetic rats subjected to ligature-induced periodontitis than in streptozotocin-diabetic rats without periodontitis. Although we observed a trend towards increasing blood glucose

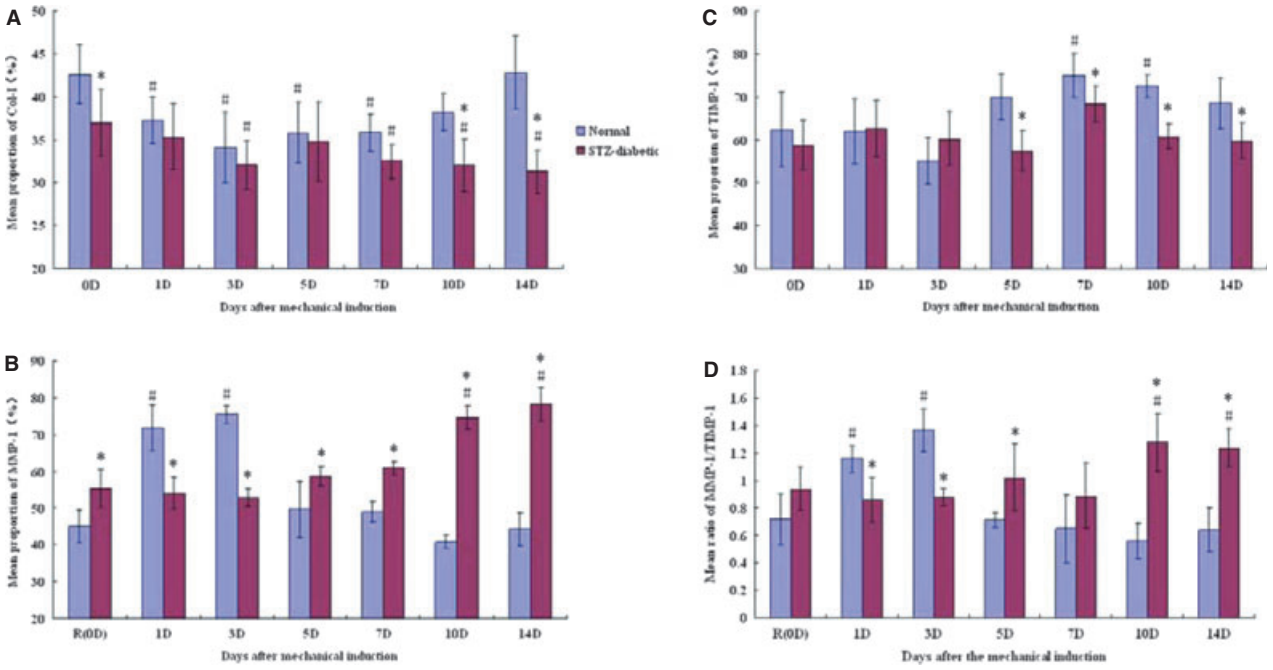



*Fig. 4.* Production of collagen-I (A), MMP-1 (B) and TIMP-1 (C), and ratio of MMP-1/TIMP-1 (D) in ND (normal) and DI (STZ-diabetic) groups after orthodontic induction. * Significant difference between the ND and DI group ($*p < 0.05$). Significant difference within group at each time point compared with the control side ($*p < 0.05$).

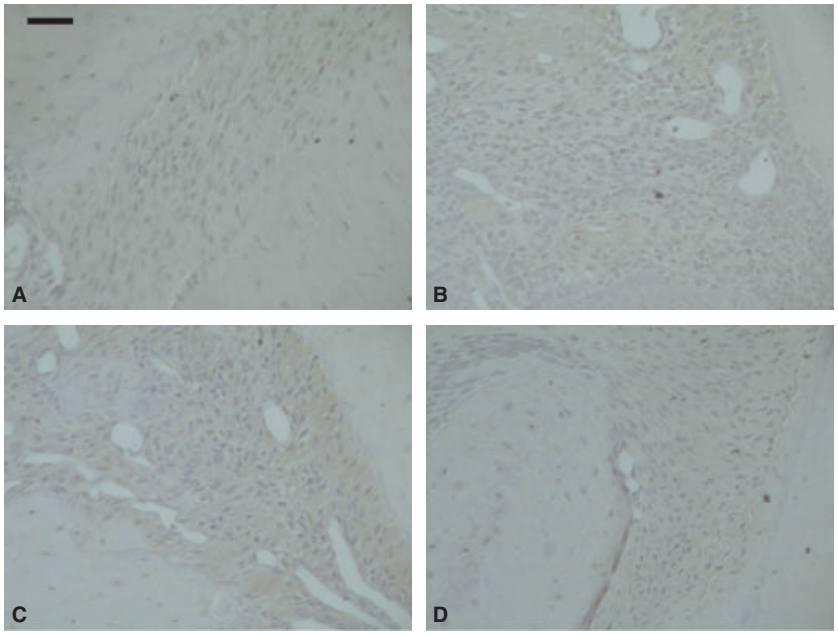



*Fig. 5.* Representative Col-I immunohistochemical pictures. (A) The contralateral control side in a ND rat on day 14. (B) The orthodontic induction side in a ND rat on day 14. (C) The contralateral control side in a DI rat on day 14. (D) The orthodontic induction side in a DI rat on day 14.

levels following induction of orthodontic force in diabetes-induced rats, the mean plasma glucose level at day 14 was not significantly different from day 0 and the day of orthodontic induction. These conflicting results may be attributed to less inflammation produced in our experiment, which only utilized mechanical force in this animal model. Further research is needed to determine whether periodontal therapy affects glycemic control.

In sum, this study described the differential production of Col-1, MMP-1 and TIMP-1 in periodontal ligament tissues under mechanical stress in diabetic and nondiabetic rats. The data indicated that diabetic rats produce more MMP, and less Col-1 and TIMP. These results suggest that diabetic patients might be more susceptible to stress-induced tissue damage. Results from this preliminary report might explain why the periodontal ligament in diabetic patients is more difficult to reconstruct after occlusal trauma. However, further study of Col-I remodeling in the diabetic periodontal ligament for longer time periods is needed to explore the reconstructive cycling of this protein in the hyperglycemic environment.

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