*Acta Odontologica Scandinavica*, 2013; 71: 1206–1215

informa
healthcare

**ORIGINAL ARTICLE**DIABETES

# Apoptosis of periodontium cells in streptozototocin- and ligature-induced experimental diabetic periodontitis in rats

YONG-WEI FU[1] & HONG-BING HE[2]

[1]*Department of Stomatology, The First People's Hospital of Lianyungang City, Lianyungang, Jiangsu Province, PR China, and* [2]*Department of Periodontology, Affiliated Stomatology Hospital of Kunming Medical College, Kunming, Yunnan Province, PR China*

**Abstract**

***Objective.*** The aim of this study was to investigate the presence of apoptosis of periodontium cells in streptozototocin- and ligature-induced experimental diabetic periodontitis in rats. ***Materials and methods.*** Sixty-two 6-week-old male Sprague-Dawley (SD) rats were randomly divided into three groups: the diabetic periodontitis group (group DP; $n = 22$), periodontitis group (group P; $n = 20$) and normal control group (group N; $n = 20$). Diabetes was induced by intraperitoneal injection of streptozototocin (STZ). Periodontitis was initiated by ligating floss around maxillary second molars. The animals were sacrificed at 3 and 6 weeks after ligature placement in the P and DP groups. Maxillary dentoalveolar segments were isolated and were prepared for morphometric analysis of alveolar bone loss (ABL) and for histological analysis. ***Results.*** ABL was significantly increased in group DP compared with group P ($p < 0.05$). The number of PDL fibroblasts, osteoblasts and osteocytes in group DP was decreased compared with group P ($p < 0.05$). Inter-group analysis revealed higher osteoclast numbers in the inflammatory area of group DP and group P when compared with group N ($p < 0.05$). Also, compared with group P, group DP had more higher osteoclast numbers ($p < 0.05$). Periodontitis and diabetic periodontitis also increased apoptosis of fibroblasts, osteoblasts and osteocytes. The percentage of these apoptotic cells was ~ 2-fold higher in group DP vs group P. ***Conclusions.*** The results of these studies suggest that diabetes may have a negative effect on the periodontium by increasing the formation of osteoclasts and enhancing apoptosis of fibroblasts, osteoblasts and osteocytes in the periodontal tissue.

**Key Words:** *animal model, apoptosis, diabetes, periodontitis*

## Introduction

Periodontal disease is induced by bacterial plaque, which accumulates on the tooth surface and stimulates a host response in the adjacent gingiva that can lead to the destruction of connective tissue and bone surrounding the tooth [1]. The progression of periodontal disease may be affected by systemic conditions, such as diabetes. It is well established that both type 1 and type 2 diabetes can increase the prevalence and severity of periodontitis. Potential pathogenic mechanisms for enhanced periodontal destruction in diabetic subjects include enhanced susceptibility to infection due to diminished neutrophil recruitment and function [2,3], a more severe inflammatory response that leads to greater tissue destruction [4], and the effect of advanced glycation end products (AGEs) on formation [5].

Apoptosis is programmed cell death that can be triggered by various signals and is characterized by well-defined morphologic changes [6,7]. Apoptosis is important as a critical mechanism for removing unwanted cells during development, as a means of preventing autoimmunity, and as part of a response to protect the host from cells that have been infected or have become tumorigenic. In pathological situations enhanced apoptosis may occur inadvertently, aggravating damage that occurs during infection or interfering with healing [8–10].

Previous studies suggest that apoptosis of inflammatory cells is involved in the pathogenesis of periodontitis [11–13]. Moreover, a body of evidence is

Correspondence: Yong-Wei Fu, Department of Stomatology, The First People's Hospital of Lianyungang City, Lianyungang 222000, Jiangsu Province, PR China. Tel: +86 0518 85606866. Fax: +86 0518 85521975-6038. E-mail: fuyongweivip@sina.com

*(Received 22 October 2011; revised 27 February 2011; accepted 5 November 2012)*

ISSN 0001-6357 print/ISSN 1502-3850 online
DOI: 10.3109/00016357.2012.757638

emerging that apoptosis plays an important role in several diabetic complications [14–16]. However, the studies about apoptosis of periodontium cells in periodontitis are scarce. Relatively little is known about the apoptosis of periodontium cells in diabetic periodontitis.

One of the characterized animal models to study diabetes in rats is the induction of type 1 diabetes by streptozotocin. The placement of ligatures around teeth to initiate periodontitis has been used in various types of animals. The ligature-induced model is sensitive to systemic effects such as smoking, diabetes and osteoporosis [17]. In the present study, we investigated the presence of apoptosis of periodontium cells in streptozotocin- and ligature-induced experimental diabetic periodontitis in rats, to test the hypothesis that apoptosis of periodontium cells may be involved in the pathogenesis of periodontitis and may represent an important mechanism through which type 1 diabetes has a negative effect on the periodontium.

## Materials and methods

### *Drugs*

Streptozotocin (STZ) was purchased from Sigma Chemical Co. (St. Louis, MO). The *in situ* Apoptosis Detection Kit was purchased from R&D Systems (Minneappolis, MN).

### *Animals*

Sixty-two 6-week-old male Sprague-Dawley (SD) rats, weighing 180–220 g, purchased from Kunming Medical Collgege Laboratory Animal Center, were housed in temperature-controlled rooms and received water and food *ad libitum*. Rats were randomly divided into three groups: the diabetic periodontitis group (Group DP, $n$ = 22), the periodontitis group (Group P, $n$ = 20) and the normal control group (Group N, $n$ = 20). The experimental procedures were approved by the Institutional Animal Research Committee at Kunming Medical College.

### *Induction of diabetes*

After adaptation (1 week) to experimental conditions, diabetic rats were fasted overnight and intraperitoneally injected with 55 mg/kg of streptozotocin freshly dissolved in 0.05 mol/l citrate buffer at pH 4.4. The periodontitis group received an intraperitoneal injection of citrate buffer alone. Rats in the normal control group were left untreated. The rats were accepted as diabetic when their fasting blood glucose exceeded 16.5 mmol/l 1 week after injection of STZ and remained >16.5 mmol/l at the time they were sacrificed.

### *Induction of periodontitis*

One week after confirmation of diabetes onset, animals in groups DP and P were anesthetized with sodium pentobarbitone (1 ml/kg, i.p.) and a nylon (000) thread ligature was surgically placed around the cervix of the second maxillary molars on both sides. The ligature was knotted on the vestibular side, so that it remained subgingivally in the palatinal side and supragingivally in the vestibular side. Animals in the normal control group were unligated. Ten animals *per* group were killed at 3 and 6 weeks after the placement of ligatures.

### *Blood glucose measurements*

At 0, 1, 3 and 5 weeks after injection of STZ or citrate buffer, glucose measurements were taken from the tail vein and measured on a commercially available glucose meter (Johnson & Johnson Company, New Brunswick, NJ).

### *Preparation of specimens*

Animals were sacrificed at 3 and 6 weeks after ligature placement. After sacrifice, the maxillae were excised immediately and separated between the middle incisors. The posterior portion of the maxillae, from the first molar to the third molar, was dissected and washed with Phosphate Buffer Saline (PBS) for 5 min then fixed in 4% paraformaldehyde. The left maxillary jaws were used to determine the degree of bone loss and the right jaws for histological analysis.

### *Measurement of alveolar bone loss*

The left jaws were defleshed and stained with 1% aqueous methylene blue to delineate the cemento-enamel junction (CEJ). Alveolar bone loss was evaluated by measuring the distance from the CEJ to the alveolar bone crest with a modification of the method of Crawford et al. [18]. In the present study, the distance was measured at six sites (buccal-mesial, buccal-central, buccal-distal, palatal-mesial, palatal-central, palatal-distal) in second molar teeth using a stereoscope (4× magnification). The mean alveolar bone loss in each experimental group was calculated from the pooled data sets, and expressed as the arithmetic means ± standard errors. The measurement of alveolar bone loss was performed by a single examiner who was masked to the study groups.

### *Histological analysis*

The right jaws were fixed in 4% paraformaldehyde for 24 h at 4°C and decalcified in 15% ethylenediaminetetra-acetic acid (EDTA) at 4°C for 3–4 weeks until decalcification was complete. The specimens were then trimmed, dehydrated in graded ethanol, cleared in toluene and embedded in paraffin. Serial 5 μm mesiodistal sections were cut sequentially. The sections were then mounted onto glass slides in preparation for rountine hematoxylin-eosin staining, tartrate-resistant acid phosphate (TRAP) staining and terminal deoxynucleotidyl transferase-mediated deoxyuridine tripphosphate nick end labeling (TUNEL).

### *Histomorphometric analysis of hematoxylin-eosin-stained sections*

The mid-interproximal region was examined in each specimen and was established by being sectioned to a level where the root canal systems in adjacent teeth were visible. The area between two adjacent molars was analyzed under light microscopy. The periodontal ligament fibroblast number in six randomly selected fields in the periodontal ligament space was counted at ×400 magnification using a micro imagery analysis system. The PDL fibroblasts were identified by their cytoplasmic and nuclear appearance in hematoxylin-eosin-stained sections. The PDL fibroblast density was expressed as PDL fibroblast number per square millimeter. Using the same method, the osteocyte density in the alveolar process between two adjacent molars was calculated. In addition, the number of osteoblasts was counted in areas of new bone formation, divided by the length of bone that contained osteoblasts. Osteoblasts were identified in hematoxylin-eosin-stained sections as cuboidal cells adjacent to bone. All data were analyzed by a blinded examiner who did not know the group to which an animal belonged and confirmed by a second examiner.

### *Histomorphometric analysis of tartrate-resistant acid-phosphate-stained (TRAP) sections*

TRAP assay on tissue sections was performed following the manufacturer's instructions. Osteoclasts were defined as TRAP-positive multinucleated cells lining bone. Using a micro-imagery analysis system, the number of Osteoclasts lining on the surface of indental alveolar bone was counted and expressed per millimeter length of bone.

### *Terminal deoxynucleotidyl transferase-mediated deoxyuridine tripphosphate nick end labeling (TUNEL) assay*

Apoptotic fibroblasts, osteoblasts and osteocytes were detected by TUNEL assay, with the use of an *in situ* apoptosis detection kit purchased from R&D Systems, performed according to the manufacturer's instructions. Briefly, after the initial deparaffinization and rehydration, slides were placed in PBS and incubated for 10 min at room temperature. Proteinase K treatment was performed for 15 min at room temperature. Then the slides were washed twice for 2 min per wash in distilled water. Endogenous peroxidase was quenched with methanol containing 3% $H_2O_2$ for 5 min. The slides were transferred into PBS for 1 min and then were immersed in the Terminal deoxynucleotidyl Transferase (TdT) labeling buffer for 5 min. Fifty microliters of labeling reaction mixture was applied to each sample and incubated for 1 h in a humidity chamber in a 37°C incubator. The reaction was stopped with TdT stop buffer and slides were washed twice in PBS for 2 min each. Finally, the slides were stained with DAB for 2–10 min at room temperature and the reaction was stopped by washing with distilled water. Slides were counterstained with methyl green, dehydrated and permanently mounted. Positive and negative control slides were prepared following the manufacturer's instructions. Apoptotic cells were defined by the characteristic of brown nuclear staining following the TUNEL assay. At high magnification (400×), TUNEL-positive fibroblasts, osteoblasts and osteocytes were counted based on morphological criteria. The data are presented as a percentage of apoptotic cells related to the total cell counts in the same field of analysis.

### *Statistical analysis*

Data are expressed as mean ± SEM. Comparisons between the three study groups were performed using one-way analysis of variance (ANOVA). In the case of significant differences among the groups, *post hoc* two-group comparisons were assessed by the Student-Newman-Keuls test. A *p*-value less than 0.05 was

Table I. Body weight (g) of all three groups.

| | Weeks after STZ administration | | | |
|---|---|---|---|---|
| | 0 | 1 week | 3 weeks | 5 weeks |
| Group N | 198.26 ± 20.32 | 235.34 ± 18.60 | 296.55 ± 15.91 | 333.37 ± 24.82 |
| Group P | 199.64 ± 16.61 | 231.68 ± 16.57 | 287.60 ± 15.70 | 318.25 ± 14.48 |
| Group DP | 198.63 ± 16.52 | 199.68 ± 17.88$^{*\dagger}$ | 207.75 ± 23.79$^{*\dagger}$ | 203.28 ± 33.57$^{*\dagger}$ |

Each value is expressed as mean ± SEM from 10–22 rats; $^{*}p < 0.05$ compared with normal control group (group N) and $^{\dagger}p < 0.05$ compared with periodontitis group (group P).

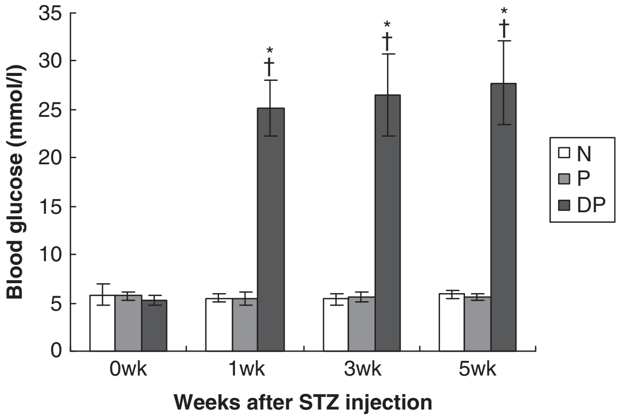

Figure 1. Blood glucose levels (mmol/L) of all three groups. Data are expressed as mean values ± SEM from 10–22 rats for each group. *$p < 0.05$, compared with the normal control group (group N). $^{\dagger}p < 0.05$, compared with the periodontitis group (group P).

considered statistically significant. Statistical analysis was performed using SPSS 13.0.

## Results

### *Body weight*

As noted in Table I, the body weight of rats in groups N and P was significantly greater than in group DP at all experimental periods. There was no significant difference between the P and the N groups.

### *Blood glucose levels*

Blood glucose levels were measured for all experimental groups (Figure 1). On week 0 of the experiment all three groups had similar blood glucose levels. After 1 week of STZ injection, blood glucose levels in the DP group were persistently above 16.5 mmol/l. Compared to the P and N groups, blood glucose levels were significantly elevated for the DP group ($p$ = 0.000). There was no significant difference between the P and the N groups.

### *Alveolar bone loss*

At both time points (3 and 6 weeks after ligature), significant differences in alveolar bone loss were found between three different groups (Figure 2). Alveolar bone loss measurements revealed significantly higher values in group DP and group P in comparison to group N ($p < 0.05$). In addition, ABL was significantly increased in group DP compared with group P ($p < 0.05$).

### *PDL fibroblast density*

To assess the potential impact of PDL fibroblast apoptosis, PDL fibroblast density was measured at 3 and 6 weeks after ligature (Figure 3A). At both time points, significant differences in PDL fibroblast density were found between three different groups. The mean PDL fibroblast density among each group is group N > group P > group DP. The PDL fibroblast density in group DP at both time points was ~ 24% lower than that in group P ($p < 0.05$).

### *Number of osteoblasts*

The number of osteoblasts in areas of new bone formation was measured. At both time ponts (3 and 6 weeks after ligature), significant differences in the numbers of osteoblasts per millimeter bone length were found between three different groups (Figure 3B). The mean numbers of osteoblasts per millimeter bone length among each group is group N > group P > group DP. The results indicated that group P had significantly higher numbers of osteoblasts per millimeter bone length compared with group DP ($p < 0.05$).

### *Number of osteocytes*

Osteocyte density was measured in the alveolar bone area. At both time points (3 and 6 weeks after ligature), significant differences in osteocyte density were found between three different groups (Figure 3C). The mean osteocyte density among each group is group N > group P > group DP.

### *Number of osteoclasts on the alveolar bone surface*

TRAP-positive osteoclasts were detected on the surface of alveolar bone (Figure 4A). At both time points (3 and 6 weeks after ligature), only a few TRAP-positive osteoclasts were detected in group N. In contrast, a large number of osteoclasts were observed in groups P and DP. In groups P and DP, the number of osteoclasts significantly increased

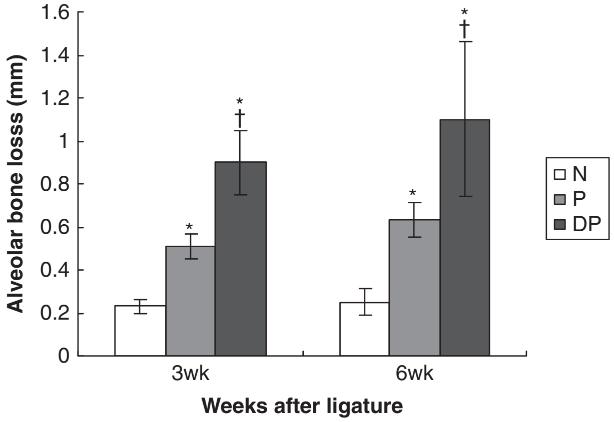

Figure 2. Alveolar bone loss detected at various time points in each group. Data represent mean values ± SEM ($n = 10$ for each group at each time-point). *$p < 0.05$ using ANOVA, compared to the normal control group (group N). $^{\dagger}p < 0.05$ using ANOVA, compared to the periodontitis group (group P).

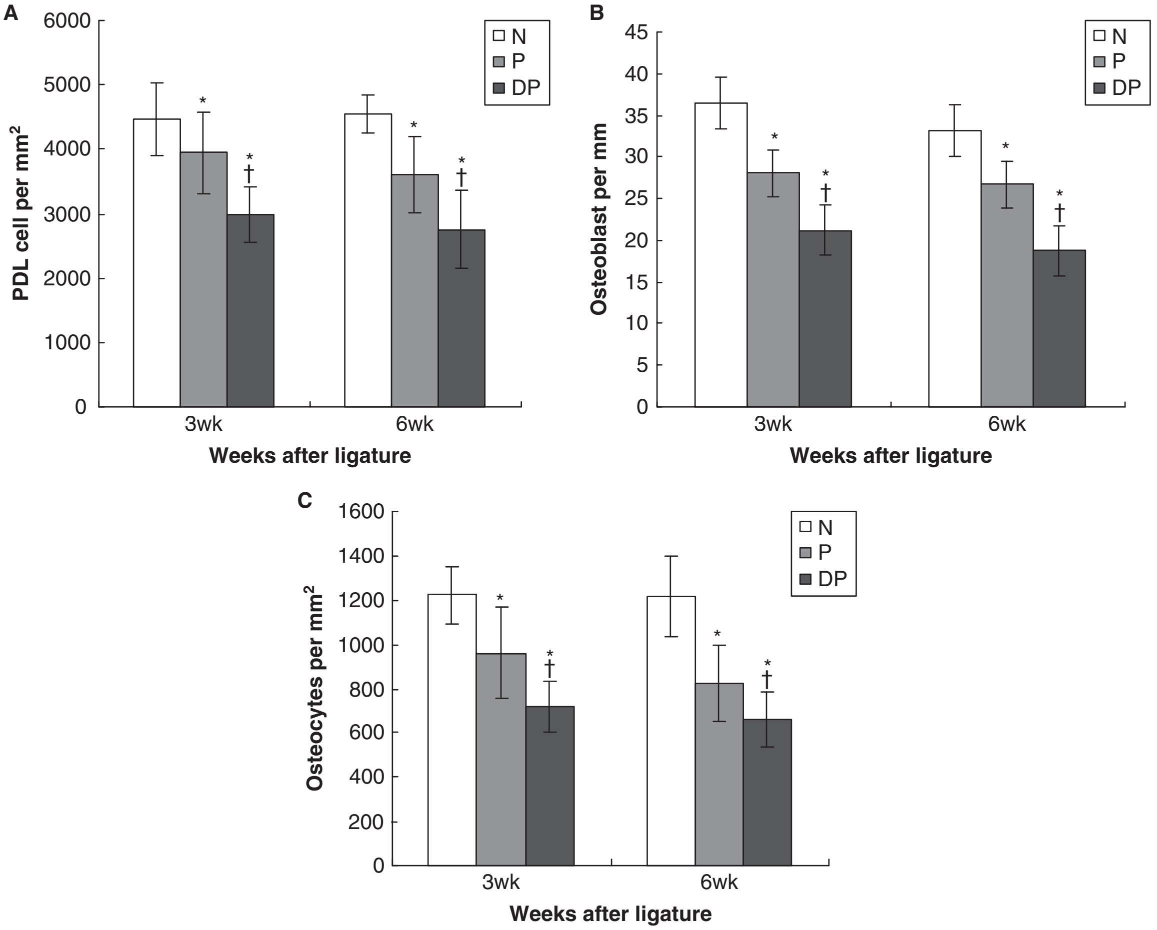

Figure 3. Diabetes decreases periodontal ligament fibroblast numbers, decreases osteoblast numbers and decreases osteocyte numbers. Numbers of periodontal ligament fibroblasts (A), osteoblasts (B) and osteocytes (C) were examined in H&E-stained sections as described in the Materials and methods section. Each value is the mean ± SEM ($n$ = 10 for each group at each time-point). *$p < 0.05$ using ANOVA, compared to the normal control group (group N). $^{\dagger}p < 0.05$ using ANOVA, compared to the periodontitis group (group P).

compared with group N. In addition, the number of osteoclasts was significantly increased in group DP compared with group P (Figure 4B).

### *Apoptosis*

To investigate the presence of apoptosis of periodontium cells in streptozototocin- and ligature-induced experimental diabetic periodontitis in rats, we detected the apoptotic cells *in situ* by TUNEL labeling. TUNEL-positive apoptotic cells exhibited a dark brown nuclear staining.

### *Apoptosis of fibroblasts*

The apoptotic fibroblasts in the area between two adjacent molars were detected. Apoptotic fibroblasts had a fusiform appearance in the TUNEL assay, distinguishing them from inflammatory cells, which have either a rounded (mononuclear cells) or multilobed appearance (PMNs). At both time points (3 and 6 weeks after ligature), dark brown apoptotic fibroblasts were found in all groups (Figure 5A). There was an apparent increase in apoptotic fibroblasts in group DP at 3 and 6 weeks when compared to group P at the same time points. In contrast, apoptotic fibroblasts were more rarely seen in group N. We then undertook quantitative analysis to measure the numbers of apoptotic fibroblasts in each group. Data were expressed as the percentage of apoptotic fibroblasts (Figure 5B). Results show that apoptosis was significantly increased in groups P and DP compared with group N. In addition, the number of apoptotic fibroblasts was significantly increased in group DP compared with group P.

### *Apoptosis of osteoblasts*

Apoptotic osteoblasts lying on bone surfaces in the area between two adjacent molars were detected. At both time points (3 and 6 weeks after ligature), dark brown apoptotic osteoblasts were found in all groups (Figure 6A). There was an apparent increase in apoptotic osteoblasts in group DP at 3 and 6 weeks when compared to group P at the same time points. In contrast, apoptotic osteoblasts were more rarely seen in group N. We then undertook quantitative analysis to measure the numbers of apoptotic osteoblasts in

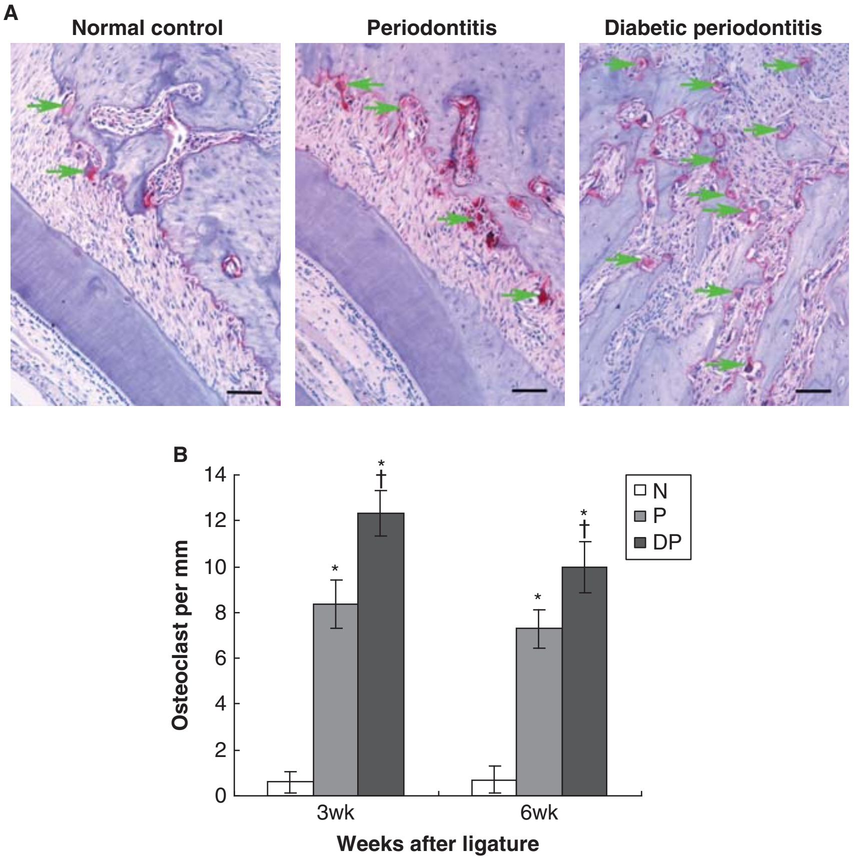

Figure 4. Diabetes enhances osteoclast formation. TRAP-positive osteoclasts were detected on the surface of alveolar bone (A). Shown are normal control group (group N), periodontitis group (group P) and diabetic periodontitis group (group DP) at week 3 after ligature. Green arrows indicate osteoclasts. Scale bars, 200 μm. Osteoclasts on the alveolar bone surface were counted (B). Data represent mean values ± SEM ($n = 10$ for each group at each time-point). $^{*}p < 0.05$ using ANOVA, compared to the normal control group (group N). $^{\dagger}p < 0.05$ using ANOVA, compared to the periodontitis group (group P).

each group. Data were expressed as the percentage of apoptotic osteoblasts (Figure 6B). Results show that apoptosis was significantly increased in groups P and DP compared with group N. In addition, the number of apoptotic osteoblasts was significantly increased in group DP compared with group P.

### *Apoptosis of osteocytes*

The apoptotic osteocytes in the area between two adjacent molars were detected. At both time points (3 and 6 weeks after ligature), dark brown apoptotic osteocytes were found in all groups (Figure 7A). There was an apparent increase in apoptotic osteocytes in group DP at 3 and 6 weeks when compared to group P at the same time points. In contrast, apoptotic osteocytes were more rarely seen in group N. We then undertook quantitative analysis to measure the numbers of apoptotic osteocytes in each group. Data were expressed as the percentage of apoptotic osteocytes (Figure 7B). Results show that apoptosis was significantly increased in groups P and DP compared with group N. In addition, the number of apoptotic osteocytes was significantly increased in group DP compared with group P.

## Discussion

The results of the experiments described in this paper demonstrate that apoptosis of periodontium cells occurs in the periodontium of rat molars. Diabetes may decrease numbers of fibroblasts, osteoblasts and osteocytes by increasing apoptosis of these cells. Thus, apoptosis of periodontium cells may be involved in the pathogenesis of periodontitis and may represent an important mechanism through which diabetes has a negative effect on the periodontium.

In the present study, the diabetic rat model was successfully reproduced by intraperitoneal injection of STZ in a single dose. It is known that streptozotocin is the most useful agent for the induction of experimental diabetes. Treatment with streptozotocin stimulates a host response that leads to destruction of $\beta$ cells, hypoinsulinemia and hyperglycemia, with many features similar to type 1 diabetes [19]. Experiments reported here indicate that streptozotocin-induced diabetes increased the severity of periodontitis in experimental rats. This is consistent with previous experimental studies [20–22]. To assess the impact of diabetes on the osteoclasts in the inflammatory area, we measured the number of osteoclasts on the surface

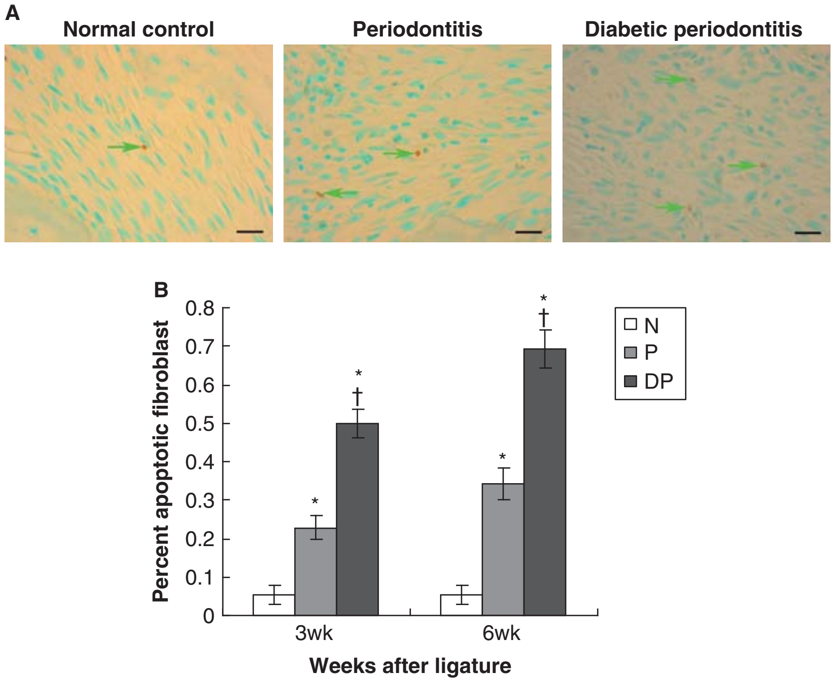

Figure 5. Diabetes increased apoptosis of fibroblasts. The apoptotic fibroblasts in the area between two adjacent molars were detected by TUNEL assay (A). Shown are the normal control group (group N), the periodontitis group (group P) and the diabetic periodontitis group (group DP) at week 3 after ligature. Green arrows indicate apoptotic fibroblasts. Scale bars, 50 µm. Apoptotic fibroblasts were counted and expressed as the percentage of apoptotic fibroblasts (B). Data represent mean values ± SEM ($n$ = 10 for each group at each time-point). $^{*}p < 0.05$ using ANOVA, compared to the normal control group (group N). $^{\dagger}p < 0.05$ using ANOVA,compared to periodontitis group (group P).

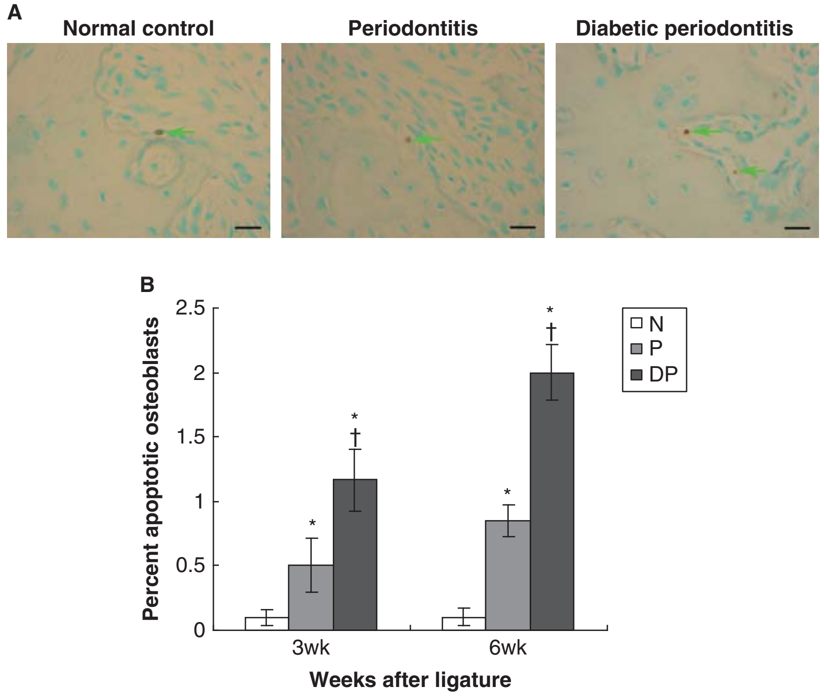

Figure 6. Diabetes increased apoptosis of osteoblasts. Apoptotic osteoblasts lying on bone surfaces in the area between two adjacent molars were detected by TUNEL assay (A). Shown are the normal control group (group N), the periodontitis group (group P) and the diabetic periodontitis group (group DP) at week 3 after ligature. Green arrows indicate apoptotic osteoblasts. Scale bars, 50 µm. Apoptotic osteoblasts were counted and expressed as the percentage of apoptotic osteoblasts (B). Data represent mean values ± SEM ($n$ = 10 for each group at each time-point). $^{*}p < 0.05$ using ANOVA, compared to the normal control group (group N). $^{\dagger}p < 0.05$ using ANOVA, compared to the periodontitis group (group P).

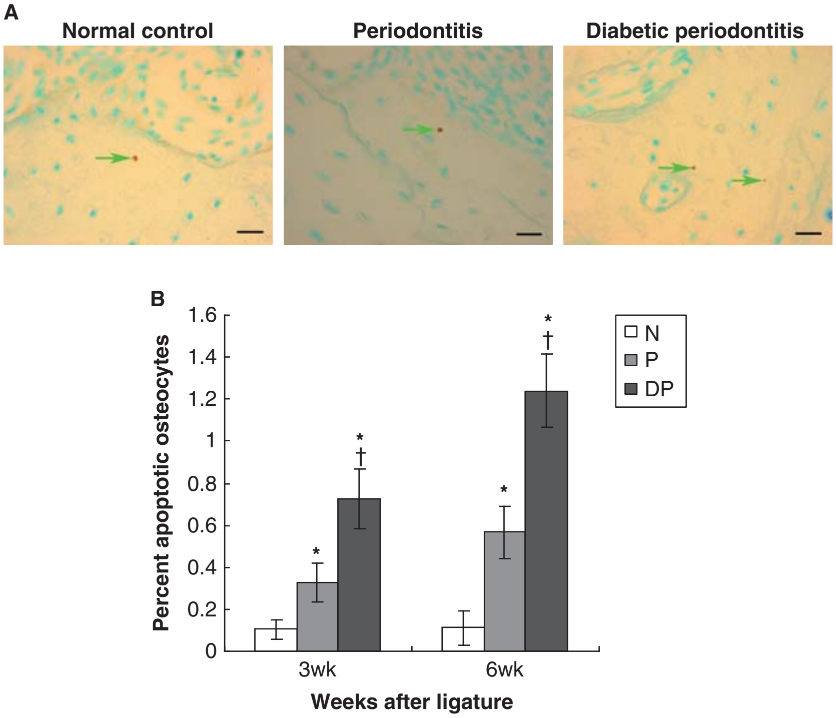

Figure 7. Diabetes increased apoptosis of osteocytes. The apoptotic osteocytes in the area between two adjacent molars were detected by TUNEL assay (A). Shown are the normal control group (group N), the periodontitis group (group P) and the diabetic periodontitis group (group DP) at week 3 after ligature. Green arrows indicate apoptotic osteocytes. Scale bars, 50 µm. Apoptotic osteocytes were counted and expressed as the percentage of apoptotic osteocytes (B). Data represent mean values ± SEM ($n = 10$ for each group at each time-point). $^*p < 0.05$ using ANOVA, compared to the normal control group (group N). $^\dagger p < 0.05$ using ANOVA, compared to the periodontitis group (group P).

of alveolar bone. The number of osteoclasts was significantly increased in group DP compared with group P. This finding suggests that diabetes enhances osteoclast formation in the inflammatory area.

A central feature of periodontitis is the remodeling of connective tissues that leads to a net loss of local soft tissues, bone and the periodontal attachment apparatus. Thus, the central issue may center not around the breakdown of tissue, but rather on the failure of adequate repair. One mechanism that might explain inadequate repair is loss of matrix-producing cells. Apoptosis of matrix-producing cells may interfere with the ability to produce enough matrix and limit repair [8,10]. PDL fibroblasts and osteoblasts are the essential cell types responsible for maintenance and repair of the periodontal ligament and alveolar bone. Osteocytes, the most abundant cells in bone, are actively involved in maintaining the bony matrix and osteocyte death is eventually followed by matrix resorption [23]. A previous study has shown that lipopolysaccharide (LPS) can indirectly induce apoptosis in osteoblasts and PDL fibroblasts *in vitro* [24]. Several studies have also indicated that some periodontal pathogens, their lipopolysaccharides and some potential virulence factors might induce apoptosis in fibroblasts [25–28]. In this study, we found that apoptosis of osteoblasts, osteocytes and PDL fibroblasts was significantly increased in groups P and DP compared with group N. The enhanced apoptosis coincided with decreased numbers of these cells. Collectively, these findings suggested that apoptosis of matrix-producing cells may be involved in the pathogenesis of periodontitis.

A body of evidence is emerging that apoptosis plays an important role in several diabetic complications. These include apoptosis of neuronal cells in diabetic neuropathy [14], diabetes enhanced myocardial apoptosis, which plays a role in cardiac pathogenesis [15] and apoptosis induced by endoplasmic reticulum stress in diabetic kidney disease [16]. In a previous study, we established that diabetes enhanced apoptosis of osteoblasts following *P. gingivalis* infection in the calvarial model [29]. In the present study, we investigated the presence of apoptosis of osteoblasts, osteocytes and PDL fibroblasts in the periodontium in streptozototocin- and ligature-induced experimental diabetic periodontitis in rats. The finding that diabetes significantly increased apoptosis of fibroblasts, osteocytes and osteoblasts raises the possibility that enhanced apoptosis may interfere with the repair of periodontal destruction. That the high rate apoptosis is functionally important is supported by the significantly decreased numbers of fibroblasts, osteoblasts and osteocytes in the diabetic rats. This agrees with previous studies reporting that diabetes enhances apoptosis of fibroblasts and osteoblasts following *P. gingivalis* infection [29,30]. Liu et al. [31] have also examined the impact of diabetes on bone resorption and formation in the ligature-induced periodontitis model in type-2 Zucker diabetic fatty (ZDF) rats. The diabetic condition also significantly increased apoptosis and decreased the number of osteoblasts and periodontal ligament fibroblasts. This supports the concept that the impact of diabetes on apoptosis in periodontitis is not dependent upon the type of diabetes, but rather is the consequence of hyperglycemia.

Increased apoptosis has been found in various organs affected by diabeties, including the eye, heart, kidney and bone [32–35]. However, the mechanism of diabetes-enhanced apoptosis is not well understood. One factor might be a prolonged inflammatory response to bacteria in diabetes [36]. A persistent infiltration of inflammatory cells coupled with advanced glycation end product (RAGE) axis caused by indirect effects of hyperglycemia could lead to sustained production of cytokines such as IL-1, IL-6 and TNF [37]. The increased TNF can amplify apoptosis by caspase-3 activation. Another mechanism of diabetes increasing apoptosis is by the production of reactive oxygen species (ROS). Persistent inflammation and hyperglycemia could cause the cellular accumulation of ROS. Oxidative stress has been shown to induce apoptosis in various types of cells [38], especially for cells in areas of active proliferation [39]. ROS have been shown to cause mitochondrial cytochrome *c* release and activation of caspase-3 [40].

In conclusion, the present study clearly demonstrates that diabetes can increase the severity of periodontitis. Accelerated alveolar bone loss was observed in diabetic rats. Increased osteoclast numbers in the inflammatory area in diabetes may contribute to the exaggerated destruction of alveolar bone. Apoptosis of periodontium cells occurs in the periodontium of rat molars. Diabetes may decrease numbers of fibroblasts, osteoblasts and osteocytes by increasing apoptosis of these cells. Thus, apoptosis of periodontium cells may be involved in the pathogenesis of periodontitis and may represent an important mechanism through which diabetes has a negative effect on the periodontium.

## Acknowledgments

The authors thank Baocai Dong for technical assistance.

***Declaration of interest:*** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## References

[1] Graves DT. The potential role of chemokines and inflammatory cytokines in periodontal disease progression. Clin Infect Dis 1999;28:482–90.

[2] Mowat A, Baum J. Chemotaxis of polymorphonuclear leukocytes from patients with diabetes mellitus. N Engl J Med 1971; 284:621–7.

[3] McMullen JA, Van Dyke TE, Horoszewicz HU, Genco RJ, Mowat A, Baum J. Neutrophil chemotaxis in individuals with advanced periodontal disease and a genetic predisposition to diabetes mellitus. J Periodontol 1981;52:167–73.

[4] Salvi GE, Yalda B, Collins JG, Jones BH, Smith FW, Arnold RR, et al. Inflammatory mediator response as a potential risk marker for periodontal diseases in insulin-dependent diabetes mellitus patients. J Periodontol 1997;68:127–35.

[5] Lalla E, Lamster IB, Feit M, Huang L, Spessot A, Qu W, et al. Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. J Clin Invest 2000;105:1117–24.

[6] Nagata S. Apoptosis by death factor. Cell 1997;88:355–65.

[7] Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998;94:491–501.

[8] Darby IA, Bisucci T, Hewitson TD, MacLellan DG. Apoptosis is increased in a model of diabetes-impaired wound healing in genetically diabetic mice. Int J Biochem Cell Biol 1997;29:191–200.

[9] Lehmann FS, Terracciano L, Carena I, Baeriswyl C, Drewe J, Tornillo L, et al. *In situ* correlation of cytokine secretion and apoptosis in Helicobacter pylori-associated gastritis. Am J Physiol 2002;283:G481–8.

[10] Rai NK, Suryabhan, Ansari M, Kumar M, Shukla VK, Tripathi K. Effect of glycaemic control on apoptosis in diabetic wounds. J Wound Care 2005;14:277–81.

[11] Gamonal J, Bascones A, Acevedo A, Blanco E, Silva A. Apoptosis in chronic adult periodontitis analyzed by *in situ* DNA breaks, electron microscopy, and immunohistochemistry. J Periodontol 2001;72:517–25.

[12] Gamonal J, Sanz M, O'Connor A, Acevedo A, Suarez I, Sanz A, et al. Delayed neutrophil apoptosis in chronic periodontitis patients. J Clin Periodontol 2003;30:616–23.

[13] Lucas H, Bartold PM, Dharmapatni AA, Holding CA, Haynes DR. Inhibition of apoptosis in periodontitis. J Dent Res 2010;89:29–33.

[14] Li ZG, Zhang W, Grunberger G, Sima AA. Hippocampal neuronal apoptosis in type 1 diabetes. Brain Res 2002;946: 221–31.

[15] Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ. Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. Diabetes 2002;51:1938–48.

[16] Liu G, Sun Y, Li Z, Song T, Wang H, Zhang Y, et al. Apoptosis induced by endoplasmic reticulum stress involved in diabetic kidney disease. Biochem Biophys Res Commun 2008;370:651–6.

[17] Graves DT, Fine D, Teng YT, Van Dyke TE, Hajishengallis G. The use of rodent models to investigate host-bacteria interactions related to periodontal diseases. J Clin Periodontol 2008;35:89–105.

[18] Crawford JM, Taubman MA, Smith DJ. The natural history of periodontal bone loss in germ-free and gnotobiotic rats infected with periodontopathic microorganisms. J Periodontal Res 1978;13:316–25.

[19] Chatzigeorgiou A, Halapas A, Kalafatakis K, Kamper E. The use of animal models in the study of diabetes mellitus. In Vivo 2009;23:245–58.

[20] Graves DT, Naguib G, Lu H, Leone C, Huse H, Krall E. Inflammation is more persistent in type 1 diabetic mice. J Dent Res 2005;84:324–8.

[21] Mahamed DA, Marleau A, Alnaeeli M, Singh b, Zhang X, Penninger JM, et al. G(-) anaerobes-reactive CD4+ T-cells trigger RANKL-mediated enhanced alveolar bone loss in diabetic NOD mice. Diabetes 2005;54:1477–86.

[22] Salvi GE, Kandylaki M, Troendle A, Persson GR, Lang NP. Experimental gingivitis in type 1 diabetics: a controlled clinical and microbiological study. J Clin Periodontol 2005; 32:310–16.

[23] Knothe Tate ML, Adamson JR, Tami AE, Bauer TW. The osteocyte. Int J Biochem Cell Biol 2004;36:1–8.

[24] Thammasitboon K, Goldring SR, Boch JA. Role of macrophages in LPS-induced osteoblast and PDL cell apoptosis. Bone 2006;38:845–52.

[25] Graves DT, Oskoui M, Volejnikova S, Naguib G, Cai S, Desta T, et al. Tumor necrosis factor modulates fibroblast apoptosis, PMN recruitment, and osteoclast formation in response to P. gingivalis infection. J Dent Res 2001;80: 1875–9.

[26] Alikhani M, Alikhani Z, Graves DT. Apoptotic effects of LPS on fibroblasts are indirectly mediated through TNFR1. J Dent Res 2004;83:671–6.

[27] Kurita-Ochiai T, Seto S, Suzuki N, Yamamoto M, Otsuka K, Abe K, et al. Butyric acid induces apoptosis in inflamed fibroblasts. J Dent Res 2008;87:51–5.

[28] Zhang JH, Dong Z, Chu L. Hydrogen sulfide induces apoptosis in human periodontium cells. J Periodontal Res 2010; 45:71–8.

[29] He H, Liu R, Desta T, Leone C, Gerstenfeld LC, Graves DT. Diabetes causes decreased osteoclastogenesis, reduced bone formation, and enhanced apoptosis of osteoblastic cells in bacteria stimulated bone loss. Endocrinology 2004;145:447–52.

[30] Liu R, Desta T, He H, Graves DT. Diabetes alters the response to bacteria by enhancing fibroblast apoptosis. Endocrinology 2004;145:2997–3003.

[31] Liu R, Bal HS, Desta T, Krothapalli N, Alyassi M, Luan Q, et al. Diabetes enhances periodontal bone loss through enhanced resorption and diminished bone formation. J Dent Res 2006;85:510–14.

[32] Joussen AM, Doehmen S, Le ML, Koizumi K, Radetzky S, Krohne TU, et al. TNF-alpha mediated apoptosis plays an important role in the development of early diabetic retinopathy and long-term histopathological alterations. Mol Vis 2009;15:1418–28.

[33] Li Y, Li Y, Feng Q, Arnold M, Peng T. Calpain activation contributes to hyperglycaemia-induced apoptosis in cardiomyocytes. Cardiovasc Res 2009;84:100–10.

[34] Velagapudi C, Bhandari BS, Abboud-Werner S, Simone S, Abboud HE, Habib SL. The tuberin/mTOR pathway promotes apoptosis of tubular epithelial cells in diabetes. J Am Soc Nephrol 2011;22:262–73.

[35] Kayal RA, Siqueira M, Alblowi J, McLean J, Krothapalli N, Faibish D, et al. TNF-alpha mediates diabetes-enhanced chondrocyte apoptosis during fracture healing and stimulates chondrocyte apoptosis through FOXO1. J Bone Miner Res 2010;25:1604–15.

[36] Wetzler C, Kämpfer H, Stallmeyer B, Pfeilschifter J, Frank S. Large and sustained induction of chemokines during impaired wound healing in the genetically diabetic mouse: prolonged persistence of neutrophils and macrophages during the late phase of repair. J Invest Dermatol 2000;115:245–53.

[37] Pierce GF. Inflammation in nonhealing diabetic wounds: the space-time continuum does matter. Am J Pathol 2001;159: 399–403.

[38] Buttke TM, Sandstrom PA. Oxidative stress as a mediator of apoptosis. Immunol Today 1994;15:7–10.

[39] Takahashi A, Aoshiba K, Nagai A. Apoptosis of wound fibroblasts induced by oxidative stress. Exp Lung Res 2002; 28:275–84.

[40] Wang GW, Klein JB, Kang YJ. Metallothionein inhibits doxorubicin-induced mitochondrial cytochrome c release and caspase-3 activation in cardiomyocytes. J Pharmacol Exp Ther 2001;298:461–8.