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ORIGINAL ARTICLE

Apoptosis: an underlying factor for accelerated periodontal disease associated with diabetes in rats

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Abstract
Objectives Diabetes mellitus (DM) is well-established risk factor for periodontal disease. DM can also lead to changes in the number of apoptotic cells in periodontal tissues. The goal of this study was to evaluate apoptosis, depending on DM, in healthy and diseased periodontal soft tissues.
Material and Methods A total of 43 adult male Sprague–Dawley rats were used in this study. Experimental periodontitis was created by placing silk ligatures around the cervices of the first mandibular molars. Experimental diabetes was induced by intraperitoneal injection of the diabetogenic agent streptozotocin (STZ). Following the induction of both experimental diseases, the animals were divided into four groups: (1) The healthy group (H) ($n=10$); (2) The diabetes group (D) ($n=10$); (3) The periodontitis group (P) ($n=11$); and (4) The diabetes and periodontitis group (DP) ($n=12$). Apoptotic cells were determined by immunohistochemistry, and the frequency of apoptotic cells was evaluated by apoptotic index score.
Results It was observed that there was less apoptosis in both the epithelial and gingival connective tissue cells of healthy diabetic tissues than in healthy tissues without diabetes. When periodontal disease existed, apoptosis increased in both the epithelial and gingival connective tissues of diabetic and non-diabetic animals.
Conclusions There may be differences in the apoptotic mechanisms in the periodontal soft tissues of diabetic and non-diabetic animals.
Clinical relevance Apoptosis may be one of the underlying factors in increased risk for periodontal disease that is associated with diabetes.

Keywords Animal model · Diabetes · Periodontal disease · Epithelium · Connective tissue

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Introduction

Chronic inflammatory periodontal disease (periodontitis) is a Gram-negative oral infection characterized by gingival inflammation, periodontal tissue destruction, alveolar bone loss and possible loss of teeth in the terminal phase [1]. Although the major etiological factor involved in periodontitis is dental microbial plaque [2], it is also known that there are many risk factors, which play an important role in the pathogenesis of periodontitis by affecting the severity and prognosis of the disease. Smoking and diabetes (DM) are well-established risk factors for periodontal disease. Genetic polymorphisms have also been investigated, although strictly speaking they cannot be generally considered risk markers or risk indicators [3]. Similarly, susceptibility to periodontal disease may be the result of defects in neutrophil function [4].

Despite the contradictory results obtained by some early studies [5], there is a direct relationship between DM and periodontitis [6]. Periodontal disease has been considered for many years to be the sixth leading complication of diabetes [7]. Epidemiological studies have indicated that diabetes mellitus (DM) increases the prevalence and severity of periodontitis, and leads to an increased risk of periodontal disease, especially when it is uncontrolled. In a comprehensive cross-sectional study with 1.426 subjects, DM and loss of attachment were positively correlated, with an odds ratio of 2.32 [8]. It was reported that Type-1 and Type-2 DM have similar risk ratios in terms of periodontal illness. In addition, despite

control of DM decreasing the prevalence of the metabolic disorders associated with the illness, it was also observed that periodontal disease cannot be prevented completely [9].

Since DM was accepted as a risk factor for periodontal pathogenesis, various scientific studies have been undertaken at the molecular level. The biological mechanism explaining the negative influence of DM on periodontal health has recently gained importance. This mechanism includes angiopathy, changes in gingival crevicular fluid, disorders of collagen metabolism, disorders of inflammatory response, changes in the subgingival microflora and genetic susceptibility [10]. Also, diabetes and related conditions such as obesity are associated with a number of physiological, nutritional and metabolic changes including hyperglycemia, production of advanced glycation end-products (AGEs) and hyperlipidemia; these changes have a number of consequences including immune-dysregulation manifested by a pronounced long-lasting inflammatory state and weakened self-limitation and resolution of immune responses [11].

Studies over the last four decades have found that animal cells are genetically programmed to destroy themselves [12]. Under standard conditions, injured or aged cells are coded for a type of cell death called apoptosis, which is not inflammatory and which requires energy, to provide cellular homeostasis. Apoptosis is the most common type of cell death, and it plays a crucial role in a great many physiological mechanisms, such as regulation of cell proliferation generated by mitosis [13]. This type of cell death is encountered in various natural processes, which are all different from each other, namely embryogenesis, the development of immune responses and recovery from inflammation. The disposal of cells by apoptosis is important for organisms in all stages of life to stay alive [14]. However, apoptosis can also be stimulated by pathological stimuli, such as exposure to radiation [15] and viral infections [16]. It has been determined that apoptosis plays an important role in the pathogenesis of Type-1 DM [17] and Type-2 DM [18]. Furthermore, as a result of diabetic complications, changes occur in the cell death mechanisms in the tissues [19]. Different results have been obtained by studies performed in different cell types affected by diabetes [20, 21]. This research subject, which has not been completely understood, remains a topic of investigation [22].

Diabetes is a systemic disease that causes numerous significant changes in the body. The disrupted energy metabolism in the tissues in diabetes can lead to changes in apoptosis [22]. These changes in the apoptosis mechanism can vary, depending on the insulin dependency of the particular tissue. In tissues such as the brain, kidney, and the liver, which are not insulin-dependent and which are capable of glucose uptake from the blood, even in the absence of insulin secretion, increases in apoptosis can be expected, whereas in insulin-dependent tissues, such as muscle, adipose tissue, connective tissue and the gingival epithelium, apoptosis can be expected to decrease, as these tissues cannot absorb glucose when insulin is not available [23].

Recent studies indicate that high glucose induces a concentration- and caspase-3-dependent increase of apoptosis in cultured human periodontal ligament fibroblasts in vitro. These results suggest a novel mechanism for the regulation of human periodontal ligament fibroblasts apoptosis by high/low glucose [24].

The goal of this study was to evaluate apoptosis, depending on DM, in periodontally/gingivally healthy gingival tissues and in gingival tissues with periodontal disease (periodontitis).

Material and methods

Animals

In this experimental study, 45 male Sprague–Dawley rats, 5–6 months old and weighing an average of 300 g were used, which were obtained from the Selcuk University Center of Experimental Medicine. All rats were housed separately in plastic cages and kept in a temperature-controlled room with a standard 12/12 h light–dark illumination cycle. All animal care and study protocols were in compliance with guidelines approved by the Animal Experiment Committee with the assignment protocol CAM 10/57-07.16.2001. Twenty-five animals were selected randomly to induce experimental periodontitis as described below.

Experimental Periodontitis

After systemic anesthesia with intraperitoneal injection of 60 mg/kg ketamine–HCl (Warner Lambert, Pfizer Inc., Istanbul, Turkey), 3.0 sterile silk ligatures were tied on the necks of mandibular first molars which were kept in position to promote microbial dental plaque accumulation and inflammation during the experimental period [25]. Two of the experimental animals died during the healing process after the operation and 23 animals could participate in the further experiment. Two weeks after the induction of the experimental periodontitis, the initial blood glucose levels of experimental animals were measured with the help of glucometer by obtaining blood from their tail vein.

Experimental diabetes

Twelve animals with established periodontitis and ten healthy animals were administered streptozotocin (STZ) in a single intraperitoneal dose (60 mg/kg, dissolved in 10 mM sodium citrate, pH 4.5, STZ; Sigma, Inc., St. Louis, MO, USA) to induce diabetes. After 7 days, diabetes was confirmed by measuring blood glucose levels (Glucometer Elite® 2000, Bayer Vital GmbH&Co. KG, Leverkusen). Animals with

blood glucose levels of 250 mg/dl were considered diabetic [26].

The same diet was provided to all of the animals for 5 weeks, and the following working groups were created: *healthy group (H)*: 10 animals without experimental diabetes and without periodontitis; *diabetic group (D)*: 10 animals with experimental diabetes and without periodontitis; *periodontitis group (P)*: 11 animals without experimental diabetes and with periodontitis; and *diabetes and periodontitis group (DP)*: 12 animals with experimental diabetes and with periodontitis.

At the end of the seventh week of the experimental period [26], the weight of the experimental animals was measured again, and the rats were sacrificed by decapitation under general anesthesia with intraperitoneal injection of 60 mg/kg ketamine–HCl (Warner Lambert, Pfizer Inc., Istanbul, Turkey) (Fig. 1). The mandibular bone of each animal was removed by dissection, including the surrounding soft tissue, and was placed in formal saline (0.1 M, pH 7.4). The obtained samples were placed on periapical dental X-ray films, and radiographs were obtained (70 kVp, 8 mA, 0.30 s, Trophy, Eastman Kodak Company, Rochester, NY, USA).

Histological Examination

The tissue samples were decalcified in 10 % ethylene-diamine-tetra-acetic acid (EDTA) for 4 weeks. At the end of the decalcification period, the samples were washed in streaming water for 24 h and were monitored through a series of graded alcohol solutions and embedded in paraffin–xylene. Paraffin sections were cut 6-μm thick in a buccolingual direction throughout the mandibular first molars and stained with hematoxylin & eosin (H&E) and Crossman's triple stain staining methods. Apoptotic changes were stained by immunohistochemical method, using an in situ apoptosis kit (TdT-FragEL kit; Oncogene Research Products, CN Biosciences, Inc. Boston, USA). During the apoptosis staining, the manufacturer's protocols were strictly followed. The prepared samples were examined with a research microscope (Leitz Laborlux-12, Esselte Leitz GmbH & Co KG, D-70469, Stuttgart), and photos were obtained digitally using the microscope, with a camera (Nikon E-400, DS-5M, Nikon Instruments Europe, Badhoevedorp) when necessary.

Histological Assessment

In the immunohistochemical assessment, the presence and distribution of apoptotic cells in the sulcular epithelial (SE), in the adjacent oral epithelial (OE) and in the gingival connective tissue (CT) regions were evaluated, counting the number of positively stained cells. The epithelial and gingival connective tissue cells were recognized by an experienced histologist on the basis of their discriminative cellular morphology. Inflammatory cells in the gingival tissues and necrotic periodontal pocket area cells were not regarded. Apoptotic index scores, ranging from 0 to 4, were given (Table 1) [27].

All of the samples were evaluated twice by the same blinded investigator, and a third evaluation was performed in cases of doubt. For the experimental evaluation, approximately 1 mm^2 of area was taken using Photoshop 7.0 software (Adobe Systems Incorporated, San Jose, CA, USA).

Statistical Methods

Animal body weights and blood glucose levels were analyzed by Wilcoxon nonparametric test. Apoptotic index scores in the epithelium and connective tissue were analyzed by Kruskal–Wallis nonparametric test and post hoc test with Bonferroni correction. Analyses were performed using a statistical software package (SPSS version 15.0, SPSS, Chicago, IL, USA).

Results

Body weight

The mean body weight of the animals, at the beginning and end of the experiment, is given in Table 2. At the end of the study, the animals in the D and DP groups experienced weight loss, and this change was found to be statistically significant ($p < 0.05$). In the H and P groups, there was a slight increase in the final weights compared to the initial weights, but this increase was not statistically significant ($p > 0.05$).

Radiological Findings

Severe periodontal disease was evident around the first molar regions on the radiographs of the animals in DP and P groups (Fig. 2).

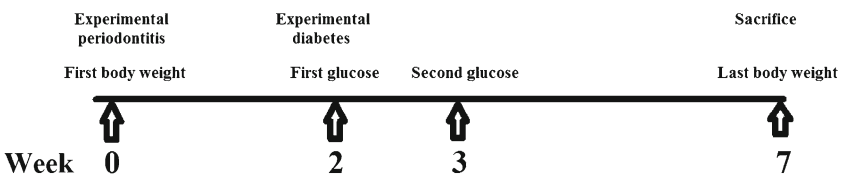

Fig. 1 Experimental design and time-course

Table 1 Apoptotic index criteria

Apoptotic index criteria
0 No positive staining cells were detected
1 The number of positively stained cells was less than 10 % of the total cells
2 The number of positively stained cells was between 10 and 25 % of the total cells
3 The number of positively stained cells was between 25 and 50 % of the total cells
4 The number of positively stained cells was greater than 50 % of the total cells

Biochemical Findings

The blood glucose levels were within the normal range in all of the animals at the beginning of the experiment. In the D and DP groups, the blood glucose levels 7 days after STZ administration were greater than 250 mg/dl in all of the animals (Table 3). The increase in blood glucose levels in the D and DP groups after 1 week was statistically significant ($p<0.05$).

Histological Findings

By H&E and Crossman's triple staining methods, under light microscopy with ×200 magnification, in the general examination of the tissues, healthy periodontal tissue was observed in the H and D groups, whereas the P and DP groups showed histological evidence of advanced periodontitis (Figs. 3 and 4).

When the samples from the D group were compared to those of the H group, no irregularities in the structure of the multilayer keratinizing squamous epithelium or histological structural defects were detected. In addition, the integrity of the basement membrane was intact. The examination of the connective tissue collagen fibers with Crossman's triple staining revealed minimal breaks and light irregularities, accompanied by significant numbers of fibroblasts. There was a mild increase in vascularization.

When the samples taken from group P were compared with those from group H, a moderate thickening of the stratified squamous epithelium was observed, and the continuity of the basement membrane was maintained. With Crossman's triple staining, in many of the samples, in the regions close to the periodontal ligament, there was significant deterioration and degeneration of collagen fibers in the connective tissue, with large numbers of irregularly located fibroblasts. Lymphocyte and macrophage infiltration into the regions close to the teeth was quite obvious. However, in the more lateral regions, this degradation and infiltration gradually decreased and disappeared.

When the samples taken from the DP group were compared to those from the H group, moderate thickening of the stratified squamous epithelium was observed, and the continuity of the basement membrane was maintained. There was irregularity of the collagen fibers and mild local fibroblast piling. There was also lymphocyte and macrophage infiltration into the regions close to the teeth (Fig. 4).

Immunohistochemical results

The tissues in the four groups were examined by immunohistochemistry for apoptotic index scores (Table 4). The tissues were split into groups of two, and the differences between them were evaluated statistically (Table 5).

The tissue samples in the H and D groups were compared separately for connective tissue and epithelial tissue (Fig. 5). The apoptosis index scores in the D group were significantly lower than those in the H group ($p<0.05$). When the H and P groups were compared, the apoptosis index scores in the P group were higher than those in the H group, but the difference in apoptosis index scores was not statistically significant. When the D and DP groups were compared, the apoptosis index scores in the DP group were found to be significantly higher than those in the D group, both in the epithelial tissue and in the connective tissue ($p<0.05$). The difference in apoptosis index scores between the P and DP groups were not statistically significant ($p>0.05$) (Tables 4 and 5).

Table 2 The mean body weights at the beginning (First), and 7th week of the experiment (Last)

Weights (g)							
Group	*n*	First Mean±st. dev.	Last Mean±st. dev.	First Median, range	Last Median, range	*Z*	*p*
H	10	343.4±38.8	349.6±44.5	346, 136	348, 134	−0.474	0.635
D	10	284.2±36.0	253.4±39.6	269, 90	240, 106	−2.805	0.005*
P	11	315.5±53.4	320.9±56.5	320, 174	322, 157	−0.044	0.965
DP	12	278.2±43.8	264.5±44.5	268, 188	253,174	−2.514	0.012*

Values are expressed as arithmetic mean±standard deviation of mean, median, and range (Wilcoxon nonparametric test)

H the healthy animals, *D* diabetic animals, *P* animals with periodontitis, *DP* animals with diabetes and periodontitis

*$p<0.05$; significant difference between First and Last measurements

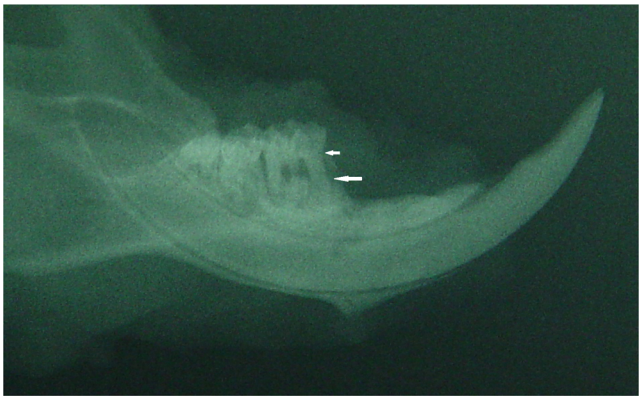

Fig. 2 Remarkable alveolar bone resorption, which is between cemento-enamel junction and the most apical alveolar bone in the ligated Group P (*short arrow* cemento-enamel junction; *long arrow* alveolar bone crest)

Discussion

In this study, apoptotic changes were investigated in periodontally/gingivally healthy soft tissues and in soft tissues with periodontal disease in diabetic and non-diabetic rats. According to the results of our research, increases in apoptosis of the epithelial and connective tissue cells were observed in periodontal disease, healthy diabetic tissues exhibited differences in apoptosis from tissues without diabetes in terms of periodontal disease, and it was observed that there was less apoptosis in both the epithelial and the gingival connective tissue cells of diabetic tissues. It was determined that when periodontal disease existed, apoptosis increased in both the epithelial and the gingival connective tissue of diabetic animals. The amount of apoptosis in these tissues was similar to that in non-diabetic animals with periodontal disease. At the end of the study, the animals in the D and DP groups experienced statistically significant weight loss ($p<0.05$). We speculate that the weight loss in these groups is associated with diabetes. In the H and P groups, there was a slight increase in the final weights compared to the initial weights, but this increase was not statistically significant ($p>0.05$).

In light of recent studies, researchers agree on the important role of apoptosis in tissue kinetics, while most of apoptotic mechanisms remain unknown. The internal and environmental factors affecting apoptosis lead to the elimination of cells in a regular and programmed manner. This process, conducted by the cell enzymes and requiring energy, is called "cell suicide" [14].

DM increases susceptibility to periodontal disease, depending on insulin secretion and/or functional disorder. Although it is widely accepted that DM affects periodontal health via different biological mechanisms, the effects on the periodontium at the molecular level remain unknown [6]. It is also known that DM causes disorders in the apoptotic mechanisms of several tissues and has different pathophysiological effects on tissues with different phenotypes and functions [20, 21]. However, the effects of DM on the physiological cell death of tissues have only recently been investigated and remain an important issue [28]. In our study, diabetic periodontal soft tissues that appeared to be periodontally/gingivally healthy exhibited less apoptosis. There was a statistically significant reduction in apoptosis, both in the epithelial tissue and in the connective tissue. This finding can be explained as adaptation of the tissues due to the reduced turnover rate [29], or it might have been due to a lack of the energy required for apoptosis [30]. Infection is known to increase apoptosis in cells. In our study, a statistically significant increase in apoptosis was found, especially in infected diabetic and non-diabetic connective tissues. This finding is interpreted as the body's immune response to infection. Microorganisms such as *Porphyromonas gingivalis* (Pg), which is a potential pathogen for periodontal diseases, cause increases in apoptosis of human periodontal ligament fibroblasts, while Pg lipopolysaccharide delays human polymorphonuclear leukocyte apoptosis [31]. Therefore, the delay in the apoptosis of polymorphonuclear leukocyte cells in chronic infection could be beneficial in terms of limiting the infection itself, but also could lead to greater periodontal destruction due to a prolonged inflammatory response. It might be claimed that the apoptosis regulation in infection is not only of bacterial origin, but also is

Table 3 The blood glucose levels in the D and DP groups at the beginning (First), and 7 days after experimental diabetes (second)

Blood glucose levels (mg/dL)							
Group	*n*	First Mean±st. dev	Second Mean±st. dev	First Median, range	Second Median, range	*Z*	*p*
D	10	87.5±7.1	436.2±127.9	88, 20	473.5, 352	−2,805	0.005*
DP	12	86.5±8.3	455.7±125.5	86.5, 25	513, 349	−3,059	0.002*

Values are expressed as arithmetic mean±standard deviation of mean, median, and range (Wilcoxon nonparametric test)

D diabetic animals, *DP* animals with diabetes and periodontitis

*$p<0.05$; significant difference between First and Second measurements

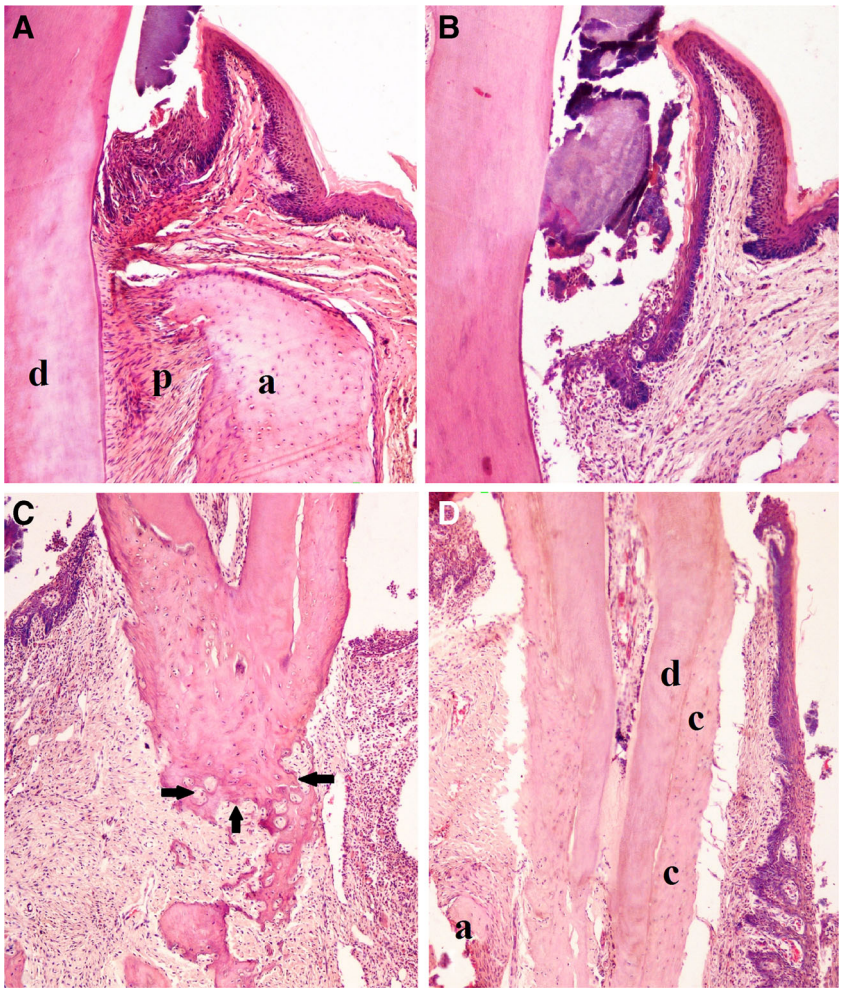

Fig. 3 Buccal-lingual sections of mandibular first molars (hematoxylin and eosin stain, original magnification ×200). **a** Histopathology of the normal periodontium of a rat in the healthy group (Group H); *a* alveolar bone, *d* dentin, *p* periodontal ligament. **b** A well-organized tooth-gingiva interface and gingival tissue with normal dimensions in the diabetic group (Group D). **c** Alveolar bone resorption and external root resorption area (*black arrows*) were observed in the periodontitis group (Group P). **d** Alveolar bone resorption in the diabetes and periodontitis group (Group DP) showed histological evidence of advanced periodontitis; *a* alveolar bone, *c* cement, *d* dentin

regulation by the host and is a measure of the defense mechanism of the host.

In our study, a very large increase in apoptosis was observed in epithelial and connective tissue in animals with both diabetes and periodontitis. This increase in apoptosis could be a specific host response to prevent the spread of the infection to deeper tissues. We can also speculate that this outcome is an enforced response to infection, and the increase in apoptosis in diabetic periodontal tissues could lead to greater periodontal destruction in the tissues in the presence of broken-down energy metabolism [30] and reduced turnover rate [29]. In addition, not only the energy metabolism but also the host defense mechanisms, such as chemotaxis, cannot perform their functions. Also, this increase in apoptosis is induced directly by periodontal pathogens, which are suspected periodontal disease agents. It might be also claimed that periodontal infection impairs diabetic periodontal soft tissue adaptation due to the reduced turnover rate and prevents limiting periodontal chronic infection itself. This hypothesis may explain susceptibility to periodontitis in diabetic tissues [31].

Some important points should be taken into consideration in studies of apoptosis. First, it must be kept in mind that although this mechanism is physiological, it can become pathological when it increases or decreases. Moreover, not only the host or host-related mechanisms but also external stimulants can trigger this mechanism [32]. In addition, apoptosis mechanisms can also be prompted directly or through a part of host defense system. Nevertheless, the same agent can cause a decrease in apoptosis in some tissues while it increases apoptosis in other tissues. For instance, while diabetic apoptosis decreases in periodontal soft tissues, intestinal mucosa, and urinary bladder cells, as adaptation of the tissues due to the reduced turnover rate in insulin-dependent tissues [20, 33], apoptosis can increase in brain, liver, or other tissues, which are not dependent on insulin [21]. In addition, while the apoptosis of some cells, such as keratinocytes, increases in the same medium and under the same conditions, the apoptosis of other body cells, such as macrophages, decreases (in infection). However, some aggressive periodontal pathogens suspected in disease, such as Aa, can reverse this state [34]. We regard this complicated state as a war. Who is firing on

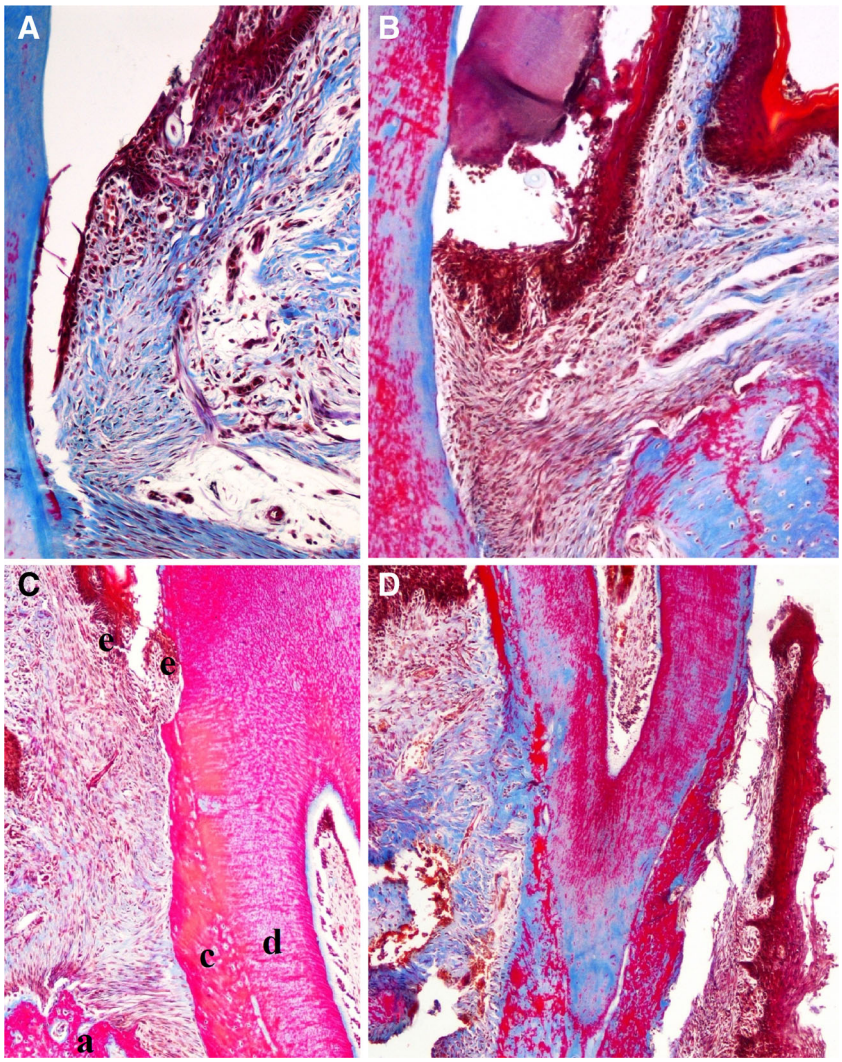

Fig. 4 Buccal-lingual sections of mandibular first molars (Crossman's triple stain, original magnification ×200). **a** Healthy structure of the periodontium of a rat in the healthy group (Group H). **b** The examination of the connective tissue collagen fibers revealed minimal breaks and light irregularities, accompanied by significant numbers of fibroblasts in the diabetic group (Group D). There was a mild increase in vascularization. **c** Significant deterioration and degeneration of collagen fibers in the connective tissue, with large numbers of irregularly located fibroblasts in the periodontitis group (Group P). Lymphocyte and macrophage infiltration into the regions close to the teeth was quite obvious; *a* alveolar bone, *c* cement, *d* dentin, *e* epithelium. **d** Moderate thickening of the stratified squamous epithelium was observed in the diabetes and periodontitis group (Group DP). There was irregularity of the collagen fibers and mild local fibroblast piling. There was also lymphocyte and macrophage infiltration into the regions close to the teeth

Table 4 Apoptotic index scores in the epithelium and connective tissue

Group	*n*	Mean	Median	Std. Dev.	Range	Minimum	Maximum
Epithelium							
H	10	2.50	2.50	1.08	3	1	4
D	10	1.20	1.00	0.92	3	0	3
P	11	3.00	3.00	0.77	2	2	4
DP	12	3.33	4.00	1.07	3	1	4
Connective tissue							
H	10	2.20	2.00	1.14	4	0	4
D	10	0.90	1.00	0.88	2	0	2
P	11	3.09	3.00	0.54	2	2	4
DP	12	3.25	4.00	0.97	2	2	4

Values are expressed as apoptotic scores arithmetic mean, median, standard deviation of mean, range, minimum apoptotic score, and maximum apoptotic score (Kruskal–Wallis nonparametric test)

H the healthy animals, *D* diabetic animals, *P* animals with periodontitis, *DP* animals with diabetes and periodontitis

whom? And why? Is the dead soldier an ally or enemy of the body? And to whom is it useful? These are some of the questions to be asked while interpreting the results of studies regarding apoptosis.

Conclusions

Based on the data obtained in this study, we speculate that there may be differences in the apoptotic mechanisms of diabetic gingival tissues, compared to the gingival tissues of non-diabetic subjects. There was a statistically significant reduction in apoptosis in diabetic periodontal soft tissues that appeared to be periodontally/gingivally healthy. This finding can be explained as adaptation of the tissues due to the reduced turnover rate in diabetes in rats. In our study, an increase in apoptosis was found, in infected diabetic and non-diabetic periodontal soft tissues. This finding is

Table 5 Post hoc test results for apoptotic index scores in the epithelium and connective tissue

Dependent variable	Group I	Group II	Mean diff.	Std. dev.	*p*	95 % confidence interval	
						LB	UB
Epithelium	H	D	1.30	0.43	0.029*	0.09	2.51
		P	−0.50	0.42	1.000	−1.68	0.68
		DP	−0.83	0.41	0.312	−1.99	0.32
	D	P	−1.80	0.42	0.001*	−2.98	−0.62
		DP	−2.13	0.41	0.000*	−3.29	−0.98
	P	DP	−0.33	0.40	1.000	−1.46	0.79
Connective Tissue	H	D	1.30	0.40	0.015*	0.18	2.42
		P	−0.89	0.39	0.176	−1.99	0.20
		DP	−1.05	038	0.058	−2.12	0.02
	D	P	−2.19	0.39	0.000*	−3.29	−1.10
		DP	−2.35	0.38	0.000*	−3.42	−1.28
	P	DP	−0.15	0.37	1.000	−1.20	0.89

Values are expressed as apoptotic scores mean difference, standard deviation, *p* value, range, lower bound (LB), and upper bound (UB) (post hoc nonparametric test with Bonferroni correction)

H the healthy animals, *D* diabetic animals, *P* animals with periodontitis, *DP* animals with diabetes and periodontitis

*$p < 0.05$; significantly different

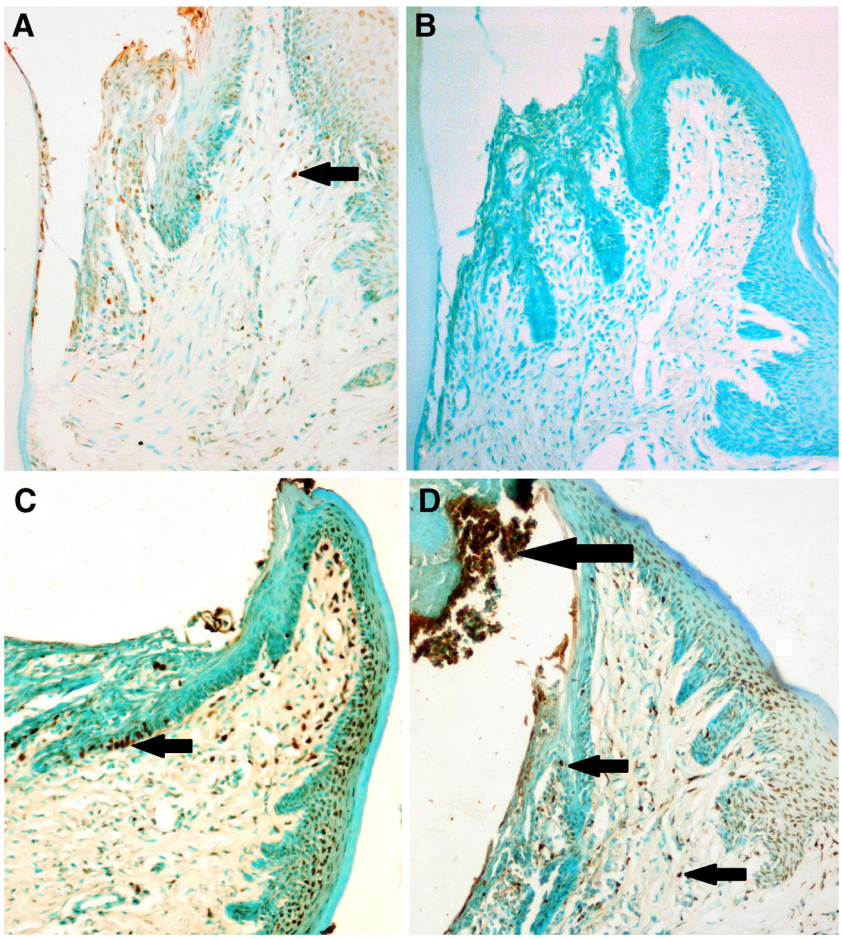

Fig. 5 Buccal-lingual sections of mandibular first molars. Apoptosis of periodontal soft tissue cells (TUNEL stain, TUNEL-positive cells are in brown, whereas nonapoptotic cells are counterstained with light blue, original magnification ×400). **a** Apoptosis in the epithelium and gingival connective tissue of a rat in the healthy group (Group H) (*arrow*, pointing to apoptotic cell). **b** No positive staining cells were detected in the diabetic group (Group D) sample. **c** Increased apoptosis in both the epithelial and gingival connective tissues in the periodontitis group (Group P) (*arrow*, point to apoptotic cells). **d** Increased apoptosis and necrotic periodontal pocket area in the diabetes and periodontitis group (Group DP) sample (*short arrows* apoptotic cells; *long arrow*, necrotic area)

interpreted as the body's immune response to infection. A very large increase in apoptosis was observed in epithelial and connective tissue in animals with both diabetes and periodontitis. This increase could be a specific host response to prevent the spread of the infection to deeper tissues, or an enforced response to infection, which could lead to greater periodontal destruction in the tissues in the presence of broken-down energy metabolism and reduced turnover rate. Within the limitations of our study, these findings in the rats' gingival tissues might also be found in humans and may explain susceptibility to periodontitis in diabetic tissues.

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Conflict of Interest "I affirm that we have no financial affiliation (e.g., employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past 3 years. Any other potential conflict of interest is disclosed."

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