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N-acetylcysteine decreases alveolar bone loss on experimental periodontitis in streptozotocin-induced diabetic rats

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Background and Objective: The purpose of this study was to evaluate the morphometric and histopathological changes associated with experimental periodontitis in diabetic rats in response to systemic administration of *N*-acetylcysteine (NAC), a sulfhydryl-containing thiol antioxidant.

Material and methods: Sixty Wistar rats were divided into six experimental groups: nonligated (NL) group; ligature-only (L) group; streptozotocin-only (STZ) group; STZ and ligature (STZ + L) group; and systemic administration of NAC and ligature (70 and 100 mg/kg body weight per day, respectively) (NAC70 and NAC100 groups). Diabetes mellitus was induced by 60 mg/kg of streptozotocin. Silk ligatures were placed at the gingival margin of the lower first molars of the mandibular quadrant. The study duration was 30 d and the animals were killed at the end of this period. Changes in alveolar bone levels were clinically measured and tissues were histopathologically examined to assess the differences among the study groups.

Results: At the end of the 30-d study period, alveolar bone loss was significantly higher in the STZ + L group compared with the other groups ($p < 0.05$). Also, alveolar bone loss in all the NAC groups was significantly lower than in the STZ + L and L groups ($p < 0.05$). The osteoblastic activity in the NAC100 group was significantly higher than in the other groups ($p < 0.05$).

Conclusion: Within the limits of this study, it can be suggested that NAC, when administered systemically, prevents alveolar bone loss in the diabetic rat model.

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Diabetes mellitus (DM) comprises a heterogeneous group of disorders characterized by altered glucose tolerance and impaired lipid and carbohydrate metabolism (1). The estimated worldwide prevalence of DM is 220.5 million, or 2.8% of the world's population (2). The incidence of this disease continues to increase at an alarming rate (1). Both type 1 and type 2 diabetes can be associated with systemic disorders, including myopathies, neuropathy, macrovascular disease and altered wound healing (3). Periodontal disease was proposed to be the sixth complication of DM, with evidence showing a correlation between poorer glycemic control and worsening

periodontal health (4). A reciprocal relationship exists between DM and periodontal disease (1,2). Periodontal infections have a significant impact on diabetic control. Conversely, DM is a significant risk factor for the development of periodontal disease and aggravates the severity of periodontal infections (1).

Oxidative stress is defined as the disturbance of the oxidant–antioxidant balance (4) and, in addition to periodontitis, is considered to be an important etiopathogenic factor in many other inflammatory diseases, including DM (5,6). As a result of oxidative stress, unbalanced radical and nonradical reactive oxygen species can damage cells by a variety of mechanisms, including peroxidation of lipid membranes, protein inactivation, induction of DNA damage and stimulation of specific signaling pathways that lead to cytokine-induced tissue damage (7,8). Reactive oxygen species are reported to be capable of inducing periodontal tissue destruction and are associated with osteoclastic bone resorption. Also, increased production of reactive oxygen species plays a very important role in the complications arising in patients with diabetes (8). Furthermore, nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) oxidase, leading to superoxide generation, is one of the major sources of reactive oxygen species production in diabetes (9,10). Taken together, scavenging or detoxification of reactive oxygen species is crucial for the maintenance of homeostasis in normal tissue and organ systems (11).

N-acetylcysteine (NAC) is a thiolic antioxidant that promotes the glutathione redox cycles as a cell-permeable glutathione precursor (12,13). Stimulation of glutathione synthesis following administration of NAC results in a greater availability of glutathione for detoxification of oxygen-derived free radicals (14). In addition to the capability of increasing cellular glutathione levels, NAC works as a direct reactive oxygen species scavenger (12). The efficacy of NAC has been investigated in clinical trials and in experimental models of various respiratory conditions, such as chronic obstructive pulmonary disease, cirrhosis, acetaminophen poisoning and diabetes (15–18). NAC decreases the concentrations of the cardiac inflammatory mediators interleukin (IL)-6 and cyclooxygenase-2, and NADPH oxidase activation, and increases the concentration of superoxide dismutase in streptozotocin-induced diabetic rat hearts (19). Also during the inflammatory process, the beneficial impact of NAC has been demonstrated in preventing the expression of lipopolysaccharide-induced inflammatory mediators (IL-1β, IL-6 and IL-8) in phagocytic cells and gingival fibroblasts, suggesting a direct link between the production of cytokine release and the generation of reactive oxygen species (20,21). As a direct result of these actions, NAC has been shown to suppress osteoclast differentiation, which leads to the prevention of bone resorption (22). However, we have previously demonstrated that systemically NAC can prevent alveolar bone loss in a dose–dependent manner in an experimental periodontitis model (23).

Based on these favorable aspects of NAC, we have hypothesized that NAC is a potent suppressor of periodontal inflammation and alveolar bone loss in diabetic rats. Therefore, the aim of the present study was to examine the validity of this hypothesis in an alveolar bone loss model in experimental periodontitis in diabetic rats.

Material and methods

Animals and experimental periodontitis model

The study protocol and experimental design were approved by the Animal Ethics Committee of Cumhuriyet University School of Medicine. In total, 60 Wistar male rats were used in the experiment. Their body weight ranged from 270 to 320 g at the beginning of the experiment. The animals were randomly divided into five groups, as follows:

- nonligated (NL) group ($n = 10$).
- ligature-only (L) group ($n = 10$).
- streptozotocin (STZ)-only (STZ) group ($n = 10$).
- STZ and ligature (STZ + L) group ($n = 10$).
- STZ and ligature plus NAC (70 mg/kg per day for 30 d) (NAC70) group ($n = 10$).
- STZ and ligature plus NAC (100 mg/kg per day for 30 d) (NAC100) group ($n = 10$).

Induction of diabetes

Diabetes was induced by a single injection of 60 mg/kg body weight streptozotocin (Sigma-Aldrich, St Louis, MO, USA), dissolved in citrate buffer (0.01 M, pH 4.5), into the jugular vein. Blood glucose levels were measured with a glycometer (IME-DC; Oberkotzau, Germany) before the procedure and 3 d after diabetes induction. A glucose level of > 300 mg/dL confirmed the presence of diabetes.

Induction of experimental periodontitis

One day after confirmation of diabetes, the rats in STZ + L, NAC70, NAC100 and L groups received ligature placement under general anesthesia (40 mg/kg of ketamine; Eczacıbasi Ilac Sanayi, Istanbul, Turkey). A 4-0 silk suture (Dogsan Sanayi, İstanbul, Turkey) was submarginally placed around the first molars of right mandibular quadrants. The sutures were checked after application, and lost or loose sutures were replaced. All ligatures were placed by the same operator (H. Ozdemir). The animals were kept in individual cages and received water and food *ad libitum*. NAC was systemically administered by gastric feeding at a rate of 0.5 mL/min. On day 30, the animals were killed and the blood samples were taken by cardiac puncture. Afterwards, the mandible was dissected free of the muscles and the soft tissue, keeping the attached gingiva intact with the bone.

Measurement of alveolar bone loss

The mandibles were stained with 1% aqueous methylene blue (Merck & Co., Inc., Whitehouse station, NJ, USA) to identify the cemento–enamel junction. The alveolar bone height was measured under a stereomicroscope (Leica Microsystems, GmbH Wetzlar, Germany)

(40× magnification) by recording the distance from the cemento–enamel junction to the alveolar bone crest. Measurements were taken at three points on both the buccal and lingual sides to quantify the alveolar bone level. A mean value for each tooth was calculated. The morphometric measurement of alveolar bone loss was performed by a single examiner (H. Ozdemır) who was unaware of the identity of samples.

Histopathological evaluation

Histological analysis was performed by a single examiner (H. Ozer) who was also blinded to the identity of samples. The mandible samples were fixed in 10% formalin and demineralized in 10% formic acid. The specimens were then dehydrated, embedded in paraffin and sectioned along the molars in a mesio-distal plane, then stained with hematoxylin and eosin and Masson's trichrome. Sections of 6 μm thickness, corresponding to the area between the first molars and the second molars where ligatures had been placed, were evaluated by light microscopy (Eclipse E 600; Nikon, Tokyo, Japan).

Inflammatory cell infiltration of the periodontal tissues; bundles of collagen fibers; existing resorption lacunae (on osteoclast surfaces) and osteoblastic activity (on forming surfaces); and the number of osteoclasts of the alveolar bone and interdental septum were measured. Inflammatory cell infiltration (ICI) was determined, by semiquantitative scoring, as no visible ICI (0), slightly visible ICI (1) and dense ICI (2). Osteoclasts were counted based on their morphology. For the evaluation of osteoblastic activity, we defined forming surfaces by the visibility of active bone-formation surfaces, which were limited by osteoid and cuboidal osteoblasts. Osteoblastic activity was determined, by semiquantitative scoring, as no activity (0), mild/moderate activity (1) and high activity (2).

Statistical analysis

Data were presented as mean ± standard deviation, or percentage, as appropriate. Osteoclast numbers and alveolar bone loss were analyzed using analysis of variance followed by Tukey's test for pairwise comparisons. The presence of ICI and osteoblastic activity were analyzed using the chi-square test. Values of $p < 0.05$ were considered statistically significant. The SPSS statistical package, version 14.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses. Alveolar bone loss and osteoclast number were determined as the expected primary outcomes of the study. The power of analysis was completed utilizing data from our previous publication (23). An alpha of 0.05 was selected for calculation. The required sample size was 10 in groups with a statistical power of 88%.

Results

The presence of the silk ligature around the first molar induced an inflammatory reaction in the periodontal tissue. The ICI in the NL group was significantly lower than the ICIs in the other groups ($p < 0.05$). Also, there was no significant difference in ICI between NL and STZ groups ($p > 0.05$). The density of ICI in the STZ + L group was higher, but not statistically significantly so, than the ICIs of the L, NAC70 and NAC100 groups.

Measurement of alveolar bone loss in the mandibular molar tooth revealed significantly higher bone-loss values in the STZ + L group compared with the other groups ($p < 0.05$) (Figs 1, 2). Alveolar bone loss in the NAC groups was significantly lower than alveolar bone loss in the STZ + L and L groups ($p < 0.05$). There was no significant difference in alveolar bone loss between the NAC70 and NAC100 groups ($p > 0.05$).

Figure 3 shows the number of osteoclasts in the study groups. There was a significantly higher number of osteoclasts in the STZ + L group than in the STZ and NL groups ($p < 0.05$). There was a higher number of osteoclasts in the STZ + L group than in the NAC70, NAC100 and L groups, but this was not statistically significant ($p > 0.05$) (Fig. 4).

No pathologic change of periodontal tissue was found in the NL group (Fig. 5A). There was no significant difference in osteoblastic activity between NL and STZ groups ($p > 0.05$). The osteoblastic activity in the NAC100 group was significantly higher than in the other groups ($p < 0.05$) (Fig. 5B and 5D). Also, the osteoblastic activity in the NAC70 group was significantly higher than in the NL, STZ and L groups ($p < 0.05$) (Fig. 5C).

Discussion

The current study demonstrated that NAC reduces alveolar bone loss in diabetic rats. Further characterization of the bone-related changes suggested that the suppression of bone loss was caused by reduced osteoclastic activity and increased osteoblastic activity. Therefore, the data support the notion that NAC acts as an antioxidant agent in the prevention of STZ and periodontitis-induced alveolar bone loss. This effect may be caused by inhibition of the oxidative burst by NAC. NAC has been shown to directly scavenge the reactive oxygen species, such as hydrogen peroxide, hydroxyl radicals and hypochlorous acid (13) and treatment with the antioxidant NAC could inhibit NADPH oxidase activation and block the release of inflammatory cytokines, such as interleukin-6 and cyclooxygenase-2, in the hearts of diabetic rats (19). Also, *in vitro* NAC has an anti-inflammatory effect by suppressing the activation of macrophages and neutrophils, attenuating leukocyte–endothelial cell adhesion and capillary leakage, and blocking the relase of inflammatory cytokines, such as tumor necrosis factor and IL-8 (24). Schmidt *et al.* (18) suggest that pretreatment of animals with the antioxidants probucol or NAC prevents the generation of thiobarbituric acid reactive substances in the diabetic gingiva. Taken together, the positive effects of NAC could be attributed to the inhibition of inflammation and bone resorption in diabetes mellitus and periodontitis.

Owing to the medical expenditure and complicated pathobiology of diabetes, research has focused on herbal medicine that might improve

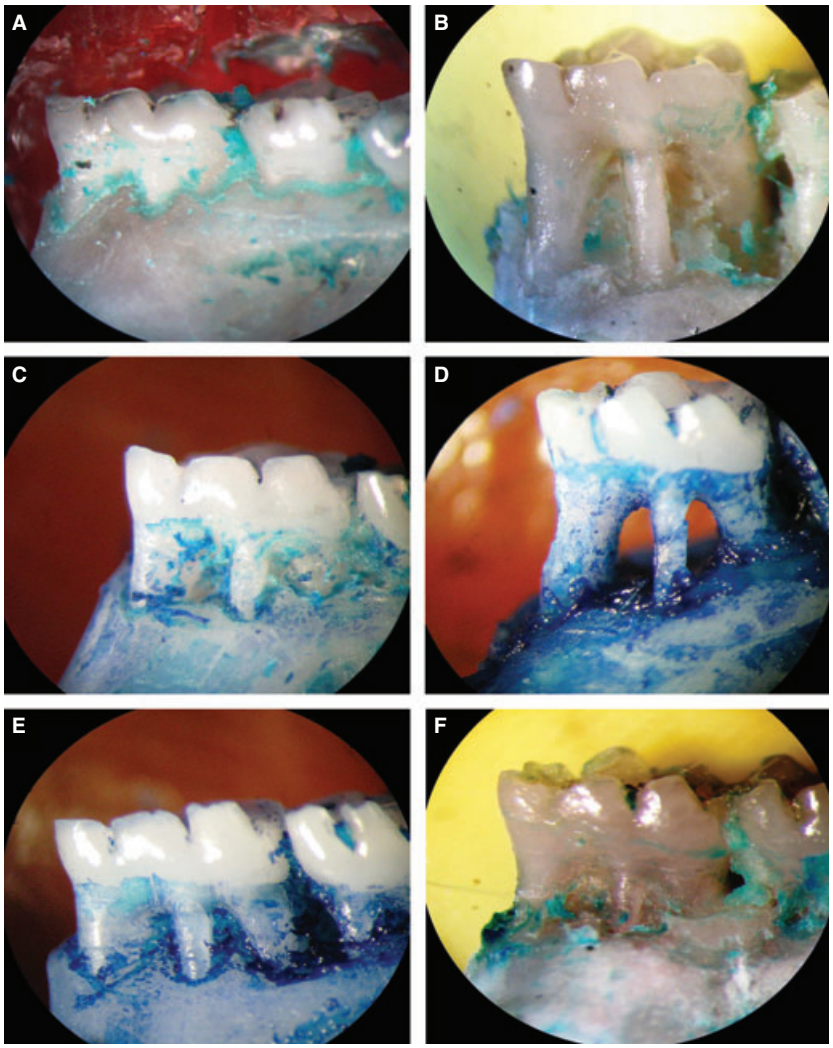

Fig. 1. Representative images of the alveolar bone loss in mandibular first molars in the control (A), ligature-only (L) (B), streptozotocin (STZ) (C), streptozotocin and ligature-only (STZ + L) (D), STZ and ligature plus *N*-acetylcysteine (NAC; 70 mg/kg per day for 30 d) (NAC70) (E) and STZ and ligature plus NAC (100 mg/kg per day for 30 d) (NAC100) (F) groups. NAC application prevents the bone loss induced by ligatures around mandibular first molars in rats.

glucose control and lower the risk of complications (25). Also, clinical evidence suggests that oxidative stress is involved in the pathogenesis of periodontitis and diabetes. To this end, in animal studies, dietary antioxidants have been extensively used in both diabetes (25–27) and periodontal disease (28–30). Our previous study (31) demonstrated that systemic administration of propolis prevented alveolar bone loss. Furthermore, the current study of Zhu *et al.* (25) demonstrated that propolis prevented the progression of STZ-induced diabetes in rats and suggested that this effect might be a result of its antioxidant ability.

Streptozotocin [2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose] is a naturally produced antibiotic from *Streptomyces achromonogenes* (32). STZ-induced hyperglycemia is a widely used experimental model (3,32,33). The generation of reactive oxygen species and the subsequent increase of local oxidative stress, DNA methylation and protein modification are suggested as the pathophysiological mechanisms of STZ-induced diabetes (32). Antioxidants were considered to be promising agents against STZ-induced diabetes. Also, the present study revealed that NAC administration decreased alveolar bone resorption as a result of the diminishing oxidative stress in STZ-induced diabetic rats with periodontitis.

NAC has been in clinical use for more than 30 years, primarily as a mucolytic agent (34) suppressing oxidative stress (13). Oral administration of NAC at doses up to 8000 mg/d is not known to cause clinically significant adverse reactions in human studies (35,36) and NAC is considered to be a safe drug with minor side effects (nausea, vomiting and heartburn), most of which are readily resolved. In our study, we used NAC at doses of 70 and 100 mg/d and found no adverse effects in the study rats.

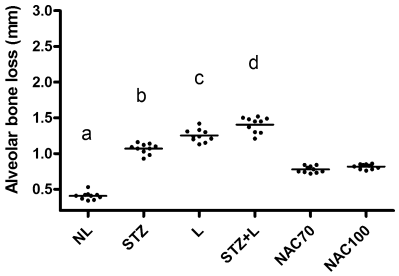

Fig. 2. Mean alveolar bone loss in the study groups. [a]$p < 0.05$ vs. the other groups; [b]$p < 0.05$ vs. ligature-only (L), streptozotocin and ligature-only (STZ + L), STZ and ligature plus *N*-acetylcysteine (NAC; (70 mg/kg per day for 30 d) (NAC70) and STZ and ligature plus NAC (100 mg/kg per day for 30 d) (NAC100) groups; [c]$p < 0.05$ vs. STZ + L, NAC70 and NAC100 groups; [d]$p < 0.05$ vs. NAC70 and NAC100 groups. NL, nonligated.

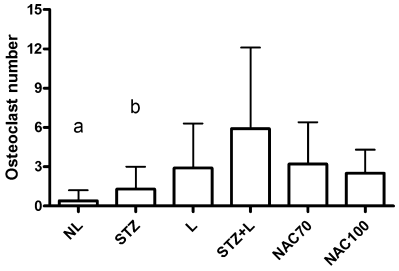

Fig. 3. Osteoclast numbers in the study groups. [a]$p < 0.05$ vs. streptozotocin and ligature-only (STZ + L) group; [b]$p < 0.05$ vs. STZ + L group. NAC70, STZ and ligature plus *N*-acetylcysteine (NAC; 70 mg/kg per day for 30 d); NAC100, STZ and ligature plus NAC (100 mg/kg per day for 30 d); NL, nonligated.

Ligature methods have been accepted as useful experimental models of periodontitis with alveolar bone resorption (37–39). This ligature favored the formation of bacterial plaque and induced an inflammatory response, reproducing human periodontal disease (33). In the present study, ligature placement on the first molar tooth caused a significant amount of bone loss and, also, the amount of bone loss was the highest in STZ-induced rats. However, for every animal model of a human disease, there are inherent limitations. Molars in rats are similar in anatomic configuration and structure to human molars, but the molars of rats are smaller, so it was difficult to perform any sort of periodontal treatment (40). A further limitation of the experimental model used is that the induced periodontitis follows an acute course, during which tissue trauma and adjacent microbial accumulation accelerate the destructive process. Such pathways of acute inflammation are likely to differ from chronic periodontitis (41).

Alveolar bone resorption is an inevitable result of periodontitis and it has also been demonstrated in diabetic conditions (33,37). Osteoclasts, in addition to phagocytes (neutrophils and macrophages), have been shown to produce reactive oxygen species and it has been suggested that these species are involved in the process of bone resorption. Although our study cannot differentiate between the cellular sources of reactive oxygen species, NAC has clearly suppressed the resorption and this effect can, at least in part, be attributed to the impact of NAC on the production of reactive oxygen species based on previous reports (42). In a study (43) that investigated the role of thiol antioxidant (NAC, ascorbate) in estrogen-deficiency bone loss in a rat model, it was found that administration of NAC for 14 d increased glutathione levels and abolished ovariectomy-induced bone loss, and, *in vitro*, NAC prevented osteoclast formation and nuclear factor-kappaB activation. In our study, the osteoclast numbers decreased in diabetic rats after the administration of NAC, but this difference did not reach statistical significance.

Several reports have demonstrated that osteoblast differentiation can be inhibited by oxidative stress, induced by exogenous stimuli such as hydrogen peroxide or xanthine/xanthine oxidase (12,44). However, in a study (45) that investigated the effects of NAC (an antioxidant), on osteoblastic differentiation in mouse calvarial cells, they found that NAC enhanced alkaline phosphatase activity, mRNA expression of osteoblast differentiation-associated genes and increased the expression of bone morphogenetic protein-2, -4 and -7. In this study, the stimulatory effect of NAC was explained by increased glutathione synthesis and down-regulation of RhoA activity. In another study (46) that evaluated the regulation of cyclooxygenase-2 expression in human osteoblastic cells by NAC, it was found that NAC could inhibit the inflammatory process involved in bone resorption by regulating cyclooxygenase-2 expression at the level of transcription. In our study, we found

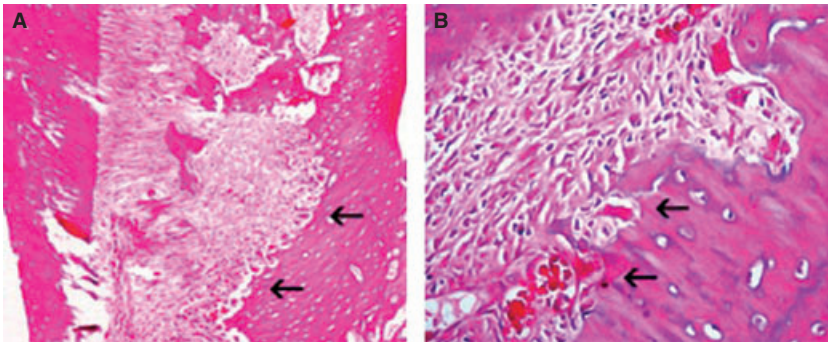

Fig. 4. (A) Alveolar bone loss after 30 d of periodontitis. Numerous osteoclasts were observed in the ligature-only (L) group (black arrows). (Hematoxylin and eosin stain; original magnification ×100.) (B) Osteoclasts with ruffled borders in the streptozotocin and ligature-only (STZ + L) group (black arrows). (Hematoxylin and eosin stain, original magnification ×400.)

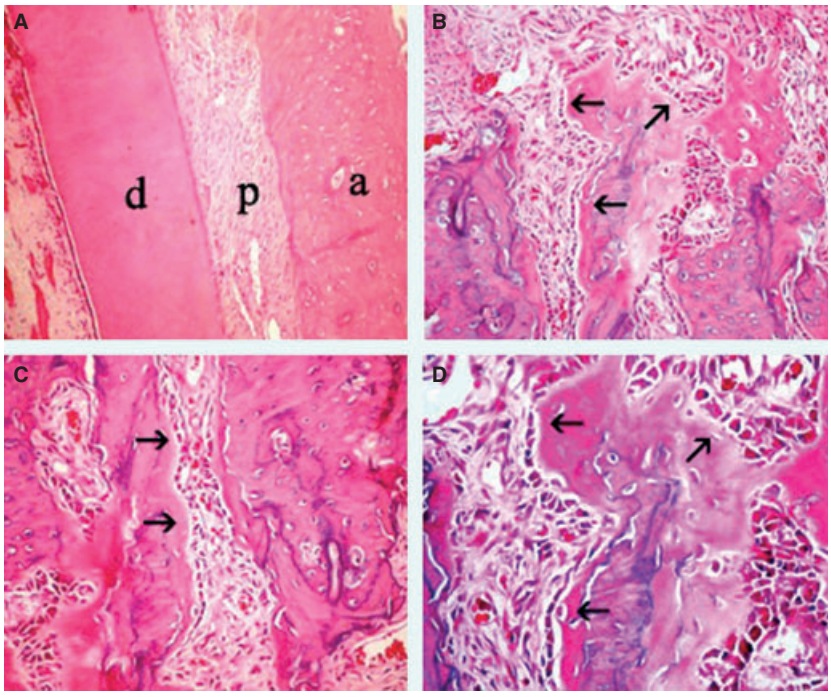

Fig. 5. (A) Histopathology of the normal periodontium of a rat. a, alveolar bone; d, dentin; p, periodontal ligament. (Hematoxylin and eosin stain, original magnification ×100). (B) and (D) Alveolar bone loss after treatment with streptozotocin and ligature plus *N*-acetylcysteine (NAC) (100 mg/kg per day for 30 d) (NAC100 group) shows increased osteoblastic activity (black arrows). (Hematoxylin and eosin stain; magnifications ×200, and ×400, respectively. (C) Osteoblastic activity (black arrows) in the STZ and ligature plus NAC (70 mg/kg per day for 30 d) (NAC70) group. (Hematoxylin and eosin stain, original magnification ×200.)

that administration of NAC increased osteoblastic activity in diabetic rats.

In conclusion, our results revealed that STZ-induced diabetes may lead to enhanced alveolar bone loss in experimental periodontitis. Furthermore, this study represents, within the inherent limitations between experimental animal and human disease interventions, evidence that systemic administration of NAC decreases alveolar bone loss in experimental periodontitis in a diabetic rat model. Although we are unable to make definitive conclusions regarding the effects of NAC on a diabetic animal model with periodontitis, the present data appear to be meaningful with regard to the beneficial effects of NAC in DM.

Conflict of interest

The authors declare that they have no conflict of interest.

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