Histometric Assessment of the Effect of Diabetes Mellitus on Experimentally Induced Periodontitis in Rats

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Abstract

Objective: The aim of this interventional animal study was to assess histologically the effect of experimental diabetes in rats with experimental periodontitis in terms of alveolar bone loss and the effect of experimental periodontitis on glucose levels in diabetes. **Materials and Methods:** Forty-seven Wistar rats were studied: 12 healthy controls (C), 10 with experimental diabetes (D), 12 with experimental diabetes and experimental periodontitis (DP) and 13 with experimental periodontitis (P). Diabetes was induced by streptozotocin injection and periodontitis was induced at the right second maxillary molar by ligation. Serum glucose levels were measured at specific time points. Sixty-one days after ligation, the rats were sacrificed. Histometric analysis assessed alveolar crest level. For ligated groups, alveolar bone loss was expressed as the difference in alveolar crest level between right and left maxillary molars. **Results:** Diabetes alone did not statistically significantly affect alveolar crest level. The combination of diabetes and periodontitis caused greater alveolar bone loss (946.1 ± 719.9 μm) than periodontitis alone (639.7 ± 294.2 μm); however, the difference did not reach statistical significance. Periodontitis did not significantly increase glucose levels in diabetic rats. The average glucose levels were 545.4 (499 - 563) and 504.5 (445 - 560) mg/dL for diabetic and diabetic ligated rats, respectively. Conclusion: Within its limits, this study demonstrated that the severity of alveolar bone loss in periodontitis was not significantly aggravated by diabetes and the serum glucose levels in diabetes were not affected by periodontitis.

Key words: Alveolar bone loss, diabetes mellitus, periodontitis, streptozotocin diabetes, rats

Introduction

Diabetes mellitus is a heterogeneous group of disorders, with the prevailing feature of high glucose levels, that affect the metabolism of carbohydrates, lipids and proteins (Mealy and Oates, 2006). Type 1 diabetes is a chronic autoimmune disease where the insulin-producing β-cells of the pancreas are destroyed, resulting in hyperglycemia because of lack of insulin secretion (Skyler, 2007). In diabetes, chronic hyperglycemia alters various tissues and organs, including the periodontium, and leads to diabetes-associated complications (Pontes Andersen *et al.*, 2007).

The importance of clinical studies in the investigation of the relationship between diabetes and periodontitis is significant. Available evidence supports the concept of increased periodontitis severity in patients with uncontrolled or poorly controlled diabetes (Kinane and Bouchard, 2008). Animal studies may explore this relationship by using methods not widely accepted and applied in humans (Kaplan and Wagner, 2006), such as histological assessment, which is

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accurate in measuring alveolar bone loss (Dumitrescu *et al.*, 2004).

Rodents are the most common animal model in experimental periodontitis and diabetes research (Pontes Andersen *et al.*, 2007). Placement of ligatures around molars (Holzhausen *et al.*, 2004; Cetinkaya *et al.*, 2007) has been used in rodent studies to induce periodontitis. Type 1 diabetes is induced chemically in rodents with alloxan (Ramamurthy *et al.*, 1972; 1974; Golub *et al.*, 1978) or streptozotocin (STZ) (Junod *et al.*, 1969; Golub *et al.*, 1978; Schneir *et al.*, 1979; Mishima *et al.*, 2002; Holzhausen *et al.*, 2004).

Alveolar bone loss and glucose levels have been compared between diabetic rats and diabetic rats with periodontitis in a short-term study by Holzhausen *et al.* (2007). There are limited data on the combination of experimental periodontitis and diabetes in terms of alveolar bone loss severity and serum glucose levels. Further investigation is required. This led the authors to examine the hypothesis of greater alveolar bone loss and higher serum glucose levels when both diseases co-exist in experimental animals for a longer period of time.

The aim of the present study was to assess histologically the effect of streptozotocin-induced diabetes on ligature-induced periodontitis in rats and to explore the effect of experimental periodontitis on serum glucose levels in diabetes.

Material and methods

Animals

Sixty adult male Wistar rats (225-250 g), bred in the laboratory of the National Center for Scientific Research "Demokritos," Athens, Greece, were used. All animals were subjected to clinical periodontal examination by using a calibrated periodontal probe (PCPUNC-15, Hu Friedy, Chicago, IL, USA). There was no animal with gingival crevicular depth > 0.5 mm, which ensured the absence of periodontitis (Verzeletti *et al.*, 2007). The animals were randomly classified into four groups of 15 each: controls (C), experimentally induced diabetes (D), experimentally induced diabetes and periodontitis (DP) and experimentally induced periodontitis (P). Subsequently, they were acclimatized to the experimental conditions for one week (Mishima *et al.*, 2002).

On day 1 of the study, all animals underwent anesthetization with intramuscular administration of ketamine hydrochloride solution (100 mg/kg body weight) (Imalgene®, 1000, Merial, Lyon, France) and xylazin (10 mg/kg body weight) (Rompun®, Bayer Health Care, Leverkusen, Germany). Thirty animals (groups D and DP) received intravenous (through the tail vein) injection of 45 mg/kg body weight streptozotocin (STZ) (Sigma, St. Louis, MO, USA) freshly dissolved in citrate buffer (10 mM, pH 4.5) for diabetes induction (Junod *et al.*, 1969). The other 30 animals (groups C and P) were injected with citrate buffer alone. Diabetes was successfully induced if serum glucose levels were > 300 mg/dL (Mishima *et al.*, 2002; Holzhausen *et al.*, 2004) up to day 5 after STZ injection. Blood samples for glucose assessment were collected through the tail vein prior to STZ or citrate buffer injection. Serum glucose levels were assessed with a glucometer (Wellion®, LINUS, AgaMatrix Inc, Salem, NH, USA). Animals with serum glucose ⩽ 300 mg/dL up to day 5 after STZ injection were excluded from the study.

On day 16 of the study, all animals were anesthetized with ketamine and xylazin. For P and DP rats, the maxillary right second molar was ligated with 4/0 silk suture (Medipac®, Kilkis, Greece) by wrapping the ligature around the tooth, carefully pushing it into the gingival sulcus and knotting it palatally. The purpose of the ligation was induction of experimental periodontitis (Gasperic *et al.*, 2002; Verzeletti *et al.*, 2007; Semenoff *et al.*, 2008). The non-ligated contralateral maxillary left second molar was used as control (Nociti *et al.*, 2000; Fernades *et al.*, 2007; Azoubel *et al.*, 2007). Control and diabetic rats did not receive suture ligatures. Ligatures remained in place in P and DP rats for 61 days to induce periodontitis. Proper position of the ligature was inspected at regular intervals three times per week (Page and Schröeder, 1982; Galvao *et al.*, 2003; Susin and Rosing, 2003; de Vasconcellos *et al.*, 2006). In cases of ligature loosening or loss it was replaced.

Serum glucose levels were examined on days 1 to 5, 16, 23, 30, 37, 44, 51, 58, 65, 72 and 77. The animals were weighed daily. On day 77, all animals were sacrificed with ketamine hydrochloride solution (200 mg/kg body weight) and pentothal solution (10%).

The animals were housed 2 per cage in stainless steel wire net cages, fed a standard rodent diet with free access to water, and exposed to a 12-h light/dark cycle. There was no bedding material on the cage floor and food was in powder form to exclude interdental foreign body or food impaction (Pontes Andersen, 2007). Glucose assessment was performed between 10.00 and 12.00 a.m. after a 12-hour fasting period. The study was conducted in accordance with guidelines approved by the Council of the American Psychological Society (1980) and the European Communities Council Directive of 24 November 1996 (86/609/EEC). The study was approved by the University of Athens Ethics and Research Committee and by the Veterinary Directorate of the Prefecture of Athens.

Histometric assessment

For each animal, the maxilla was dissected out and fixed in 10% neutral formalin for 48 hours. Specimens were gradually demineralized in 0.5 M neutral EDTA solution for 1 month. Then, they were thoroughly rinsed in tap water, sectioned into right and left

Table 1. Comparison of weight and weight change by rat group using one-way ANOVA and Kruskal-Wallis test[§].

Weight (g)	C n = 12	D n = 10	DP n = 12	P n = 13	F-test	*p*-value
W_1	266.2 ± 25.2	263.8 ± 18.5	274.5 ± 24.5	266.4 ± 16.3	0.55	0.65
W_{77}	306.3 ± 25.8	238.2 ± 46.0*†	259.3 ± 39.2*‡	304.9 ± 29.5†‡	10.4	< 0.001
W_m	279.4 ± 18.7	229.1 ± 32.0*†	243.08 ± 30.23†‡	276.62 ± 18.4†‡	11.1	< 0.001
ΔW_{77-1}	40.2 ± 27.1	-25.6 ± 33.8*†	-15.17 ± 36.54†‡	38.54 ± 33.08†‡	12.95	< 0.001
ΔW_{16-1}	-10 (-15, 8)	-38 (-56, -30)*†	-36 (-43, -20)*‡	-2 (-10, 14)†‡	31.16[§]	0.001
ΔW_{77-16}	46.2 ± 17.4	18.05 ± 25.7*	23.00 ± 23.24	38.92 ± 21.02	4.16	0.01
W_{Em}	283.6 ± 19.0	227.8 ± 35.1*†	242.33 ± 32.81*‡	280.38 ± 20.17†‡	11.8	< 0.001

*Significantly different from C group ($p < 0.05$); †significant difference between D and P groups ($p < 0.05$); ‡significant difference between DP and P groups ($p < 0.05$). C, controls; D, diabetes; P, periodontitis; DP, diabetes and periodontitis; W_1, day 1 weight; W_{77}, day 77 weight; W_m, average weight; ΔW_{77-1}, difference between final and baseline weight; ΔW_{16-1}, difference in weight between day 16 and baseline; ΔW_{77-16}, difference between final weight and weight at day 16; W_{Em}, average weight from day 16

maxillary parts and embedded in paraffin cubes. Semi-serial longitudinal mesio-distal maxillary sections parallel to the tooth long axis (Carranza *et al.*, 1971; Breivik *et al.*, 2001; Niikura *et al.*, 2005; Toker *et al*, 2008) were sliced at 6 μm thickness. Sections were counterstained with haematoxylin and eosin, examined and photographed under a light microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan) equipped with a digital camera (Nikon DS-2MW, Nikon, Tokyo, Japan) and transferred for image analysis into a computer, equipped with the Image Pro Plus 5.1 Program (Media Cybernetics, Bethesda, MD, USA). A central section from the molar middle area, in which the proximal tooth surfaces and alveolar bone coronal part were properly imaged, was studied (Breivik *et al.*, 2001).

The distance between the cemento-enamel junction (CEJ) and alveolar crest (AC) (Semenoff *et al.*, 2009) was measured mesially and distally on each second molar with the aid of the Image Pro Plus 5.1 Program. CEJ-AC distance (or alveolar crest level) for each second molar was the average of mesial and distal CEJ-AC distances for a given molar. The CEJ-AC distance (H) for each animal belonging to non-ligated groups (C and D) was the average of the CEJ-AC distance for the right (H_R) and left (H_L) second molar. The difference in the CEJ-AC distance between the right (H_R) and left (H_L) second molar was calculated for each animal (ΔH_{R-L}). ΔH_{R-L} expressed the alveolar bone loss for each animal belonging to the ligated groups (P and DP). The animal was the unit of measurement for the alveolar bone loss in the present study. Data are presented as distances in micrometers (μm).

Each CEJ-AC distance was measured twice (2 weeks apart) by the same examiner, who was masked to animal group and glucose levels. The measurement documented was the average of these two scorings. In most sites, the scorings were identical. In about 10% of the sites, the two scorings differed by 2 to 3%.

Arithmetic determinations

The following calculations were made: the difference between final and baseline weight (ΔW_{77-1}) = day 77 weight (W_{77}) –day 1 weight (W_1); difference in weight between day 16 and baseline (ΔW_{16-1} = day 16 weight (W_{16}) – W_1; difference between final weight and weight at day 16 ($\Delta W_{77-16} = W_{77} - W_{16}$); average weight ($W_m$ = average of daily weight throughout the study period); average weight from day 16 and after (W_{Em} = average of daily weight from day 16 to 77); average glucose levels (G_m = average of glucose levels at days 16, 23, 30, 37, 44, 51, 58, 65,72 and 77); CEJ-AC distance for each animal of groups C and D ($H = H_R+H_L/2$); difference in CEJ-AC distance between right and left second molar for each animal ($\Delta H_{R-L} = H_R-H_L$).

Statistical analysis

Mean values and standard deviations were calculated for weight, glucose levels and histological parameters at different time points. One-way analysis of variance (ANOVA) was used for comparison of these measurements among the four groups (C, D, P, DP) at each time point. Two-by-two comparisons were assessed using the *t*-test with Bonferroni correction. Comparisons within groups between different time points were examined using the *t*-test for paired data. When the normality assumption was not met, median and 1st and 3rd quartiles were used for data description. Non-parametric Mann-Whitney and Kruskal-Wallis tests were applied for comparisons between two or more than two groups at different time points, respectively. Wilcoxon matched pairs signed ranks tests were also used for within groups comparisons across time. Commercially available statistical software (Stata

Table 2. Comparison of glucose levels by rat group using one-way ANOVA[§] and Kruskal-Wallis test.

Glucose (mg/dL)	C n = 12	D n = 10	DP n = 12	P n = 13	F-test	*p*-value
G_1	101.8 ± 18.8	114.4 ± 14.0	118.7 ± 28.0	111.9 ± 14.1	1.55 [§]	0.211
G_5	118 (105, 126)	396 (327, 476)[*†]	383.5 (285, 426.5)[*‡]	129(122,137)[†‡]	34.27	0.0001
G_{16}	129 (123, 140.5)	520.5 (449, 601)[*†]	496 (420, 570)[*‡]	141(125,146)[†‡]	34.45	0.0001
G_{23}	135.5 (109, 159.5)	520.5 (388, 601)[*†]	473.5 (315, 591.5)[*‡]	132(117,155)[†‡]	34.27	0.0001
G_{44}	134.5 (131, 141.5)	601 (558, 601)[*†]	543.5 (476, 601)[*‡]	115(97,126)[†‡]	36.28	0.0001
G_{77}	157 (136.5, 184.5)	500 (458, 601)[*†]	525 (362.5, 601)[*‡]	128 (124, 151)[†‡]	29.81	0.0001
G_m	139.5(135.5, 147)	545.5 (499, 563)[*†]	504.5 (445, 560)[*‡]	137(131, 145)[†‡]	34.64	0.0001

*Statistically significant difference from C group ($p < 0.001$); [†]statistically significant difference between D and P groups ($p < 0.001$); [‡]statistically significant difference between DP and P groups ($p < 0.001$); C, controls; D, diabetes; P, periodontitis; DP, diabetes and periodontitis; G_1, day 1 glucose; G_5, day 5 glucose; G_{16}, day 16 glucose; G_{23}, day 23 glucose; G_{44}, day 44 glucose; G_{77}, day 77 glucose; G_m, average glucose levels.

Table 3. Weight values at three time points: analysis by rat group and comparison among the time points using paired data *t*-test.

Group	W_1 Mean ± SD (g)	W_{16} Mean ± SD (g)	W_{77} Mean ± SD (g)	W_{77} vs W_1 *p*-value	W_{16} vs W_1 *p*-value	W_{77} vs W_{16} *p*-value
C	266.2 ± 25.2	260.2 ± 5.1	306.3 ± 25.8	0.0003	0.15	< 0.001
D	263.8 ± 18.5	220.02 ± 7.8	238.2 ± 46.0	0.04	< 0.001	0.054
DP	274.5 ± 24.5	236.33 ± 8.03	259.3 ± 39.2	0.18	0.0004	0.006
P	266.4 ± 16.3	266.00 ± 4.66	304.9 ± 29.5	0.01	0.92	< 0.001

"C,controls; D, diabetes; P, periodontitis; DP, diabetes and periodontitis; W1, day 1 weight; W16, day 16 weight; W77, day 77 weight."

9.0, Stata, College Station, TX, USA) was used. The level of statistical significance was set at 5% ($p = 0.05$).

Results

Diabetes was induced in all STZ-injected rats. Among the 60 rats initially included in the study, 47 rats survived. The number of surviving rats and the survival rates were 12, 10, 12 and 13 rats, and 80, 67, 80 and 87% for C, D, DP and P groups, respectively. Survival rate did not significantly differ among groups (x^2 test = 1.87, $p = 0.60$). All groups were statistically comparable in baseline weight (W_1, *Table 1*) and glucose (G_1, *Table 2*). For the 47 rats, mean W_1 and G_1 were 267.85 ± 21.17 g and 111.57 ± 20.25 mg/dL, respectively.

Body weight

There were statistically significant differences among the four groups in W_{77}, W_m, W_{Em}, $\Delta W_{77\text{-}1}$, $\Delta W_{16\text{-}1}$ and $\Delta W_{77\text{-}16}$. W_{77}, W_m, WE_m, $\Delta W_{77\text{-}1}$ and $\Delta W_{16\text{-}1}$ statistically significantly differed between C and D, C and DP, P and D and P and DP groups. W_{77} was statistically significantly higher than W_1 for C and P groups, and statistically significantly lower than W_1 for the D group. W_{16} was statistically significantly lower than W_1 for the D and DP groups. W_{77} was statistically significantly greater than W_{16} for C, DP and P groups (*Table 3*).

Serum glucose levels

Groups D and DP were markedly hyperglycemic during the experimental period. G_m levels were increased 2.90 times in D and 2.62 times in DP rats compared to controls. Spontaneous recovery from the diabetic state was not noted after STZ injection. Final glucose (G_{77}) and G_m levels were significantly lower for C compared to D and DP, and for P compared to D and DP groups (*Table 2*). Glucose did not differ between D and DP and between C and P groups at all given times. The comparison of glucose levels among groups at days 1, 5 (G_5), 16 (G_{16}), 23 (G_{23}), 44 (G_{44}) and 77 appears in *Table 3* (data not shown for the other time points).

Distance between cemento-enamel junction and alveolar crest

The median CEJ-AC distance (H) was 428 μm (370-531) and 508 μm (443-551) for the C and D groups, respectively. Groups C and D did not statistically significantly differ in H (Mann Whitney z = 1.45, $p = 0.15$). Statistically significant differences were not found in the comparison of CEJ-AC distance at the left second molar (H_L) among the four groups (Kruskal-Wallis $x^2 = 3.40$, $p = 0.33$; *Table 4*). Comparison of the median difference in CEJ-AC distance between right and left second molars ($\Delta H_{R\text{-}L}$) among groups revealed statistically significant differences (F = 13.46, $p <$

Table 4. Comparison of the distance between cementoenamel junction (CEJ) and alveolar crest (AC) by rat group using one-way ANOVA[§] and Kruskal-Wallis test.

Distance (μm)	C n = 12	D n = 10	DP n = 12	P n = 13	F-test	*p*-value
H_L	432 (272, 504)	506 (396, 556)	438 (353, 550)	520.5 (399, 616)	3.40	0.33
ΔH_{R-L}	9.0 ± 291.9	41.1 ± 153.3[†‡]	946.1 ± 719.9[*‡]	639.7 ± 294.2[*†]	13.46[§]	< 0.001

*Statistically significantly different from C group ($p < 0.005$); [†]statistically significant difference between D and P groups ($p = 0.01$); [‡]statistically significant difference between DP and D groups ($p < 0.001$); C, controls; D, diabetes; P, periodontitis; DP, diabetes and periodontitis; H_L, CEJ-AC distance at the left second molar; ΔH_{R-L}, difference in CEJ-AC distance between right and left second molar for each animal.

0.001). ΔH_{R-L} was greater for P than C and D, and for DP compared to C, D and P groups. All differences in ΔH_{R-L} were statistically significant, except for the difference between DP and P ($p = 0.49$). Groups C and D did not differ significantly in ΔH_{R-L} (*Table 4*).

Discussion

The present interventional animal study explored the effect of STZ-induced diabetes on ligature-induced periodontitis in Wistar rats in terms of alveolar bone loss and the effect of periodontitis superimposed on diabetes on serum glucose levels. For these purposes 10 diabetic, 13 ligated, 12 diabetic ligated and 12 healthy control animals were studied concerning alveolar crest level, weight changes and glucose levels.

Diabetes reduced body weight, which is in accordance with findings by Schneir *et al.* (1979) and Mishima *et al.* (2002). The finding that diabetic rats lost significant weight soon after STZ, whereas they gained weight thereafter implies that the initial weight loss could be attributed to stress imposed on the animal by the newly induced diabetes. The addition of periodontitis to diabetes did not affect weight. Periodontitis did not affect weight, which is consistent with the findings of Cetinkaya *et al.* (2008), but not those of Bezerra *et al.* (2000). The present similar weight gain in periodontitis and control rats agrees with the results of Dumitrescu *et al.* (2004). The present similar weight patterns between ligated and control groups imply that the former rats did not eat less in general than the latter ones. Potent discomfort at the ligated site might have altered masticatory patterns, but not sufficiently to decrease food intake.

Validation of the diabetes model was evidenced by hyperglycemia after the 45 mg/kg STZ injection, sustained hyperglycemia and high G_m. This agrees with previous results of the diabetogenic action of a STZ injection of relatively low dose in Wistar rats, specifically of 40 (Mishima *et al.*, 2002), 45 (Junod *et al.*, 1969) or 50 mg/kg (Holzhausen *et al.*, 2004; Gomes *et al.,* 2009). Additionally, the results of the diabetogenic effect of STZ are consistent with those in other animal models (Hageman and Buschard, 1994). Periodontitis superimposed on diabetes did not affect glucose levels, because G_m and glucose levels at all given times did not differ between diabetic and ligated diabetic animals. The present findings contradict results by Holzhausen *et al.* (2004) of significantly higher glucose levels in ligated diabetic rats than in diabetic rats. Similarly, periodontitis alone did not alter glucose levels in this study, as demonstrated by the absence of differences between ligated and control rats, which is in line with observations by Gomes *et al.* (2009).

Most of the information on the alveolar crest level in the healthy Wistar rat periodontium comes indirectly from controlled studies, where controls did not receive any manipulation. Direct arithmetic comparisons of alveolar crest level in healthy Wistar rats among the studies are difficult mainly because of differences in study design, such as assessment method, tooth area and points of reference. However, the present median proximal alveolar crest level in maxillary second molars [428 (370-531) μm] is similar to the mean histologic palatal value reported by Grevstad (1993) for maxillary first molars (500 ± 87 μm, range 350-600 μm) and to the mean radiographic value reported by Cetinkaya *et al.* (2007) for mandibular first molars (0.372 ± 0.028 mm). The wide range in crest level values in the present study might partly be attributed to inter-rat variability. Root proximities might be another factor accounting for this. Relatively high standard deviation has been reported by Holzhausen *et al.* (2004) for the radiographically assessed alveolar crest level in the diabetic group (0.12 mm), which was their non-ligated control, and by Gomes *et al.* (2009) for the radiographically assessed alveolar crest level in the healthy control group (0.4 mm).

The subgingival placement of 4.0 silk suture ligature around the maxillary second molar was successful in inducing periodontitis in Wistar rats. This is in line with previous studies (Gasperic *et al.*, 2002; Verzeletti *et al.*, 2007; Semenoff *et al.*, 2008). The threshold CEJ-AC distance for alveolar bone loss in the Wistar rat periodontitis model has not been suggested in the literature. A ligated group was considered to present periodontitis when the mean value of the differences between right and left alveolar crest level was significantly greater than in the control group. Ligating one molar per animal and calculating the

ligature-induced bone loss for each animal as the difference in crest level between the ligated molar and the non-ligated contralateral molar overcame problems that continuous tooth movement in an occlusal direction might arise in rodent studies. Occlusal tooth wear/attrition, occlusal tooth movement and CEJ-AC increase with time (Page and Schröeder, 1982) and seem to be indigenous limitations of the rodent periodontitis model. Alveolar bone loss has been expressed as the difference between right and left sites in previous studies (Azoubel *et al.*, 2007; Di Paola *et al.*, 2007) as well.

Uncontrolled diabetes alone had no effect on crest level, since it was similar for diabetic animals and healthy controls. Therefore, diabetic rats were not more likely to present with normally occurring periodontitis than non-diabetic controls. The present similarity contradicts findings by Gomes *et al.* (2009) for significantly greater radiographic CEJ-AC distance in the diabetic group compared to the healthy control group. Uncontrolled diabetes superimposed on periodontitis did not significantly affect the bone loss severity. Specifically, the combination of diabetes and periodontitis led to greater bone loss than periodontitis alone; however, the difference did not reach statistical significance. The statistical significance of the difference in crest level was stronger between diabetic ligated and control groups ($p < 0.001$) than between ligated and control groups ($p = 0.004$), as well as between the diabetic ligated and diabetic groups ($p < 0.001$) than between ligated and diabetic groups ($p = 0.01$). The present finding on statistically similar bone loss severity between diabetic ligated and ligated animals is in accordance with the radiographic findings of Holzhausen *et al.* (2004) 30 days after ligation. However, it contradicts findings by Gomes *et al.* (2009) of significantly greater radiographic bone loss for the former than the latter group 30 days after ligation.

In the present study, the animals were ligated for 61 days as in studies by Cetinkaya *et al.* (2007) and Semenoff *et al.* (2008). However, bone loss in ligated rats has been detected at 30 days (Holzhausen *et al.*, 2004; Fernandes *et al.*, 2007) and even at 7 days (Holzhausen *et al.*, 2004) after ligation. Tying ligatures around molars enhances bacterial growth. Because of the minute anatomic structures of the periodontium in rodents small amounts of bacteria in the gingival sulcus are likely to trigger host response reactions (Pontes Andersen et *al.*, 2007). This might explain why periodontitis develops relatively soon after ligation in rodents. A longer ligation time was selected in this study compared to most previous experiments based on past findings on increasing bone loss over time in ligated rats (Holzhausen *et al.*, 2002; 2004; Gomes *et al.*, 2009) and in order to prolong the sustained hyperglycemia.

Sustained hyperglycemia influences the periodontium by altering microvasculature, increasing collagenase activity, prolonging the inflammatory response and impairing host defense against microbial challenges, wound healing and new bone formation (Golub *et al.*, 1978; Ramamurthy and Golub, 1983; Inoue *et al.*, 1997; Hayashi *et al.*, 1999; Lalla *et al.*, 2001; Mishima *et al.*, 2002; Graves *et al.*, 2004; 2006; Liu *et al.*, 2006; Pontes Andersen *et al.*, 2007). It leads to indirect damage through formation of advanced glycation end products, to direct cellular damage through stimulation of various intracellular pathways, and to modifications in the periodontal environment (Pontes Andersen *et al.*, 2007). The duration of sustained hyperglycemia seems important for the development of tissue alterations. Long lasting high glucose levels are a strong risk factor for diabetes-associated complications (The Diabetes Control and Complications Trial Research Group, 1994) and increase the possibility of severe systemic complications (Noack *et al.*, 2000). It has been suggested that periodontitis severity might be greater in diabetics with more severe systemic complications (American Academy of Periodontology, 2000). Taking into consideration that diabetes-associated complications require considerable time to develop, a longer experimentation time might reveal statistically significant differences in alveolar crest level between diabetic ligated and ligated animals. Superimposition of diabetes on periodontitis for a longer time might show that diabetes significantly accentuates alveolar bone loss severity.

Within its limits, this study demonstrated that co-existence of STZ-induced diabetes and ligature-induced periodontitis did not significantly increase the severity of alveolar bone loss observed in rats with periodontitis alone or the serum glucose levels measured in rats with diabetes alone.

References

American Academy of Periodontology. Position Paper. Diabetes and Periodontal diseases. *Journal of Periodontology* 2000; **71**:664-678.

Azoubel, M.C., Menezes, A.M., Bezerra, D., Oria, D., Ribeiro, R.A. and Brito, G.A. Comparison of etoricoxib and indomethacin for the treatment of experimental periodontitis in rats. *Brazilian Journal of Medical and Biological Research* 2007; **40**:117-125.

Bezerra, M.M., de Lima, V., Alencar, V.B., *et al.* Selective cyclooxygenase-2 inhibition prevents alveolar bone loss in experimental periodontitis in rats. *Journal of Periodontology* 2000; **71**:1009-1014.

Breivik, T., Thrane, P.S., Gjermo, P. and Fonnum, F. Postnatal glutamate-induced central nervous system lesions alter periodontal disease susceptibility in adult Wistar rats. *Journal of Clinical Periodontology* 2001; **28**:904-909.

Carranza, F.A. Jr., Simes, R.J., Mayo, J. and Cabrini, R.L. Histometric evaluation of periodontal bone loss in rats. I. The effect of marginal irritation, systemic irradiation and trauma from occlusion. *Journal of Periodontal Research* 1971; **6**:65-72.

Cetinkaya, B.O., Keles, G.C., Ayas, B. and Gurgor, P. Effects of risedronate on alveolar bone loss and angiogenesis: a stereologic study in rats. *Journal of Periodontology* 2008; **79**:1950-1961.

Cetinkaya, B.O., Keles, G.C., Ayas, B., Sakallioglu, E.E. and Acikgoz, G. The expression of vascular endothelial growth factor in a rat model at destruction and healing stages of periodontal disease. *Journal of Periodontology* 2007; **78**:1129-1135.

de Vasconcellos, L.M., Ricardo, L.H., Balducci, I., de Vasconcellos, L.G. and Carvalho, Y.R. Histological analysis of effects of 24% EDTA gel for nonsurgical treatment of periodontal tissues. *Journal of Oral Science* 2006; **48**:207-214.

Di Paola, R., Mazzon, E., Muià, C., *et al.* 5-aminoisoquinolin-1 (2H)-one, a water-soluble poly (ADP-ribose) polymerase (PARP) inhibitor reduces the evolution of experimental periodontitis in rats. *Journal of Clinical Periodontology* 2007; **34**:95-102.

Dumitrescu, A.L., Abd-El-Aleem, S., Morales-Aza, B. and Donaldson, L.F. A model of periodontitis in the rat: effect of lipopolysaccharide on bone resorption, osteoclast activity, and local peptidergic innervation. *Journal of Clinical Periodontology* 2004; **31**:596-603.

Fernandes, M.I., Gaio, E.J., Oppermann, R.V., Rados, P.V. and Rosing, C.K. Comparison of histometric and morphometric analyses of bone height in ligature-induced periodontitis in rats. *Brazilian Oral Research* 2007; **21**:216-221.

Galvao, M.P., Chapper, A., Rosing, C.K., Ferreira, M.B. and de Souza, M.A. Methodological considerations on descriptive studies of induced periodontal diseases in rats. *Pesquisa Odontologica Brasileira* 2003; **17**:56-62.

Gasperic, R., Stiblar-Martincic, D. and Skaleric, U. Influence of restraint stress on ligature-induced periodontitis in rats. *European Journal of Oral Sciences* 2002; **110**:125-129.

Golub, L.M., Schneir, M. and Ramamurthy, N.S. Enhanced collagenase activity in diabetic rat gingiva: *In vitro* and *in vivo* evidence. *Journal of Dental Research* 1978; **57**:520-525.

Gomes, D.A., Spolidorio, D.M., Pepato, M.T., *et al.* Effect of induced diabetes mellitus on alveolar bone loss after 30 days of ligature-induced periodontal disease. *Journal of the International Academy of Periodontology* 2009; **11**:188-192.

Graves, D.T., Al-Mashat, H. and Liu, R. Evidence that diabetes mellitus aggravates periodontal diseases and modifies the response to an oral pathogen in animal models. *Compendium of Continuing Education in Dentistry* 2004; **25** Suppl 1:38-45.

Graves, D.T., Liu, R., Alikhani, M., Al-Mashat, H. and Trackman, P.C. Diabetes-enhanced inflammation and apoptosis – impact on periodontal pathology. *Journal of Dental Research* 2006; **85**:15-21.

Grevstad, H.J. Doxycycline prevents root resorption and alveolar bone loss in rats after periodontal surgery. *Scandinavian Journal of Dental Research* 1993; **101**:287-291.

Hageman, I. and Buschard, K. Diabetic animal models. In: Svendsen, P. and Jay, J. (Eds): *Handbook of Laboratory Animal Science*. Boca Raton, FL: CRP Press, 1994; vol. II, pp 103-123.

Hayashi, A., Shinohara, M. and Ohura, K. Effect of insulin on naturally occurring gingivitis in rats with diabetes. *Journal of Osaka Dental University* 1999; **33**:1-7.

Holzhausen, M., Garcia, D.F., Pepato, M.T. and Marcantonio, E. Jr. The influence of short-term diabetes mellitus and insulin therapy on alveolar bone loss in rats. *Journal of Periodontal Research* 2004; **39**:188-193.

Holzhausen, M., Rossa, Junior C., Marcantonio, Junior E., Nassar, P.O., Spolidorio, D.M. and Spolidorio, L.C. Effect of selective cyclooxygenase-2 inhibition on the development of ligature-induced periodontitis in rats. *Journal of Clinical Periodontology* 2002; **73**:1030-1036.

Inoue, H., Shinohara, M. and Ohura, K. The effect of leukocyte function of streptozotocin-induced diabetes in naturally occurring gingivitis rat. *Journal of Osaka Dental University* 1997; **31**:47-54.

Junod, A., Lambert, A.E., Stauffacher, W. and Renold, A.E. Diabetogenic action of streptozotocin: relationship of dose to metabolic response. *Journal of Clinical Investigation* 1969; **48**:2129-2139.

Kaplan, J.R. and Wagner, J.D. Type 2 diabetes – An introduction to the development and use of animal models. *Institute for Laboratory Animal Research Journal* 2006; **47**:181-185.

Kinane, D. and Bouchard, P. Periodontal diseases and health: Consensus report of the sixth European workshop on periodontology. *Journal of Clinical Periodontology* 2008; **35** Suppl 8:333-337.

Lalla, E., Lamster, I.B., Stern, D.M. and Schmidt, A.M. Receptor for advanced glycation end products, inflammation, and accelerated periodontal disease in diabetes: mechanisms and insights into therapeutic modalities. *Annals of Periodontology* 2001; **6**:113-118.

Liu, R., Bal, H.S., Desta, T., *et al.* Diabetes enhances periodontal bone loss through enhanced resorption and diminished bone formation. *Journal of Dental Research* 2006; **85**:510-514.

Mealey, B.L. and Oates, T.W. Diabetes mellitus and periodontal diseases. *Journal of Periodontology* 2006; **77**:1289-1303.

Mishima, N., Sahara, N., Shirakawa, M. and Ozawa, H. Effect of streptozotocin-induced diabetes mellitus on alveolar bone deposition in the rat. *Archives of Oral Biology* 2002; **47**:843-849.

Niikura, K., Takeshita, N. and Chida, N. A novel inhibitor of vacuolar ATPase, FR202126, prevents alveolar bone destruction in experimental periodontitis in rats. *Journal of Toxicological Sciences* 2005; **30**:297-304.

Noack, B., Jachmann, I., Roscher, S., *et al.* Metabolic diseases and their possible link to risk indicators of periodontitis. *Journal of Periodontology* 2000; **71**:898-903.

Nociti, F.H. Jr., Nogueira-Filho, G.R., Primo, M.T., *et al.* The influence of nicotine on the bone loss rate in ligature-induced periodontitis. A histometric study in rats. *Journal of Periodontology* 2000; **71**:1460-1464.

Page, R.C. and Schröeder, H.E. *Periodontitis in Man and Other Animals*. New York: Karger, 1982, 58-106.

Pontes Andersen, C.C., Flyvbjerg, A., Buschard, K. and Holmstrup, P. Relationship between periodontitis and diabetes: lessons from rodent studies. *Journal of Periodontology* 2007; **78**:1264-1275.

Ramamurthy, N.S., Zebrowski E.J. and Golub, L.M. The effect of alloxan diabetes on gingival collagen metabolism in rats. *Archives of Oral Biology* 1972; **17**:1551-1560.

Ramamurthy, N.S., Zebrowski E. J. and Golub, L.M. Insulin reversal of alloxan-diabetes induced changes in gingival collagen metabolism of the rat. *Journal of Periodontal Research* 1974; **9**:199-206.

Ramamurthy, N.S. and Golub, L.M. Diabetes increases collagenase activity in extracts of rat gingiva and skin. *Journal of Periodontal Research* 1983; **18**:23-30.

Schneir, M., Bowesox, J., Ramamurthy, N., *et al.* Response of rat connective tissues to streptozotocin-diabetes. Tissue-specific effects on collagen metabolism. *Biochimica et Biophysica Acta* 1979; **583**:95-102

Semenoff, T.A., Semenoff-Segundo, A., Bosco, A.F., Nagata, M.J., Garcia, V.G. and Biasoli, E.R. Histometric analysis of ligature-induced periodontitis in rats: a comparison of histological section planes. *Journal of Applied Oral Sciences* 2008; **16**:251-256.

Skyler, J.S. Prediction and prevention of type 1 diabetes: progress, problems and prospects. *Clinical Pharmacology and Therapeutics* 2007; **81**:768-771.

Susin, C. and Rosing, C.K. Effect of variable moderate chronic stress on ligature-induced periodontal disease in Wistar rats. *Acta Odontologica Scandinavica* 2003; **61**:273-277.

The Diabetes Control and Complications Trial Research Group. The relationship of glycemic exposure (HbA1c) to the risk of development and progression of retinopathy in the diabetes control and complications trial. *Diabetes* 1995; **44**:968-983.

Toker, H., Ozan, F., Ozer, H., Ozdemir, H., Eren, K. and Yeler, H. A morphometric and histopathologic evaluation of the effects of propolis on alveolar bone loss in experimental periodontitis in rats. *Journal of Periodontology* 2008; **79**:1089-1094.

Verzeletti, G.N., Gaio, E.J. and Rosing, C.K. Effect of methotrexate on alveolar bone loss in experimental periodontitis in Wistar rats. *Acta Odontologica Scandinavica* 2007; **65**:348-351.