

## Diabetes and its effects on dental pulp

Orlando Catanzaro<sup>1,2</sup>), Damian Dziubecki<sup>3</sup>), Lorena Calvo Lauria<sup>2</sup>),  
Camila Martinez Ceron<sup>3</sup>) and Ricardo R. Rodriguez<sup>1</sup>)

<sup>1</sup>)Medical School and School of Dentistry, University Del Salvador, Buenos Aires, Argentina

<sup>2</sup>)Dental School, University Argentina John F. Kennedy, Buenos Aires, Argentina

<sup>3</sup>)School of Biochemistry, University Argentina John F. Kennedy, Buenos Aires, Argentina

(Received 10 January and accepted 9 September 2006)

**Abstract:** Uncontrolled or poorly controlled diabetes mellitus may be a risk factor for the development of oral complications. The objective of this investigation was to determine the effect of diabetes mellitus progression on inflammatory and structural components of dental pulp. Male Wistar rats were given a single injection of Streptozotocin (STZ), and induction of diabetes was confirmed 24 h later. Dental pulp tissue samples were taken from central incisors and molars of diabetic rats 30 and 90 days after the STZ treatment. Plasma glucose levels in diabetic rats 30 and 90 days after STZ treatment were significantly increased when compared to control rats ( $P < 0.001$ ). Nitrite and kallikrein levels in dental pulp tissue were higher in diabetic rats 30 days after STZ treatment than in controls, while only nitrite were decreased 90 after of STZ treatment. Myeloperoxidase activity showed changes 30 and 90 days after STZ injection when compared to controls. The activity of alkaline phosphatase showed significant changes 30 and 90 days after STZ treatment. On the other hand the concentration of collagen was decreased in diabetic rats 30 and 90 days after STZ injection. These results suggest that diabetes is a critical factor that has profound effects upon oral tissues, resulting in expression of inflammatory mediators and modifications of structural components of dental pulp. (J. Oral Sci. 48, 195-199, 2006)

Keywords: diabetes; dental pulp; kallikrein; myeloperoxidase; collagen.

---

---

### Introduction

Diabetes mellitus (DM) is a complex multisystemic disorder characterized by a relative or absolute insufficiency of insulin secretion or resistance to the metabolic action of insulin on target tissues (1). The metabolic consequence of the disease is hyperglycemia with widespread complications and damage, in particular, microvascular disease (microangiopathy), macrovascular disease (macroangiopathy) with accelerated arteriosclerosis, neuropathy involving neuromuscular dysfunction and decreased resistance to infection (2). In uncontrolled or poorly controlled diabetic the inflammatory reaction is more acute because of vascular leakage and cell dehiscence. Long-standing diabetes has been demonstrated to result in angiopathy and thickened basement membrane in dental pulp vessels (3). Studies of rats with streptozotocin-induced diabetes have been revealed a marked reduction in plasma blood flow in dental pulp (4). It has been shown that increased blood glucose levels lead to the development of thickened basement membranes in blood vessels, particularly capillaries. Animal models of uncontrolled diabetes mellitus show periapical and periodontal lesions (5,6). Although several studies have investigated the effects of diabetes on dental pulp, the effects of short and long-standing diabetes on dental pulp components such as nitrites, kallikrein, alkaline phosphatase, myeloperoxidase and collagen are still unclear. The objective of the present investigation, therefore, was to determine whether diabetes resulted in pulpal inflammation and over-production or unbalanced production of chemical mediators.

## Materials and Methods

Experiments were carried out on male Wistar rats (200-250 g) that had free access to water and regular chow. The protocol of this study was approved by the Institutional Committee of Medical School of USAL. Diabetes was induced by a single intravenous injection of Streptozotocin (STZ) at 60 mg/kg body wt. dissolved in citrate buffer (pH 4.5). Control rats received vehicle only (citrate buffer). After 24 h diabetes was confirmed by determination of the tail-vein plasma glucose level. The animals were randomly assigned to the following groups: control (C) ( $n = 8$ ), blood glucose 5.5 mm/l; 30-day diabetic rats (DI) ( $n = 10$ ), blood glucose 15.51 mm/l; 90-day diabetic rats (DII) ( $n = 10$ ), blood glucose 33.5 mm/l. At predetermined times points (30 or 90 days), the rats were anesthetized with ether, and dental pulp was extracted from the lower incisors and molars under magnification.

Each tooth was immediately split with a small mallet and pulp was extracted with small forceps. The collected pulp tissue samples were washed extensively with 50 mM Tris-HCl (pH 7.5) at 4°C. Tissue was drained on filter paper, weighed (6-8 mg) and then homogenized in cold 10mM Tris-HCl, pH 8.2. The homogenate was centrifuged (10,000 x g) at 4°C for 30 min and the supernatant saved for biochemical determinations. Protein was determined according to the method of Lowry et al. (7). Nitrites were determined with the Griess reagent (Promega System) according to Green et al. (8) [0.1% NED solution; 1% sulfanidiamine (SDF) in 5% perchloric acid, 0.1 M Na nitrite standard]. Samples (100 µl) were preincubated with SFD for 5-10 min at room temperature, then 50 µl of NED solution was added and incubation was continued for 30 min at 60°C. A nitrite standard curve was prepared. Absorbance was measured at 520 nm. Tissue kallikrein was determined by incubating 100 µl of pulp homogenized with 1 mM p-Val-Leu-Arg-paranitroaniline (S 2266, Chromogenic, Sweden), 20 mM SBTI and 700 µl of Tris buffer (0.2 M, pH 9.5) for 60 min at 37°C. The reaction was stopped with 100 µl of 50% acetic acid, the mixture was centrifuged for 15 min, and the sample was determined by absorbance at 405 nm. The enzyme activity was expressed as µmol 10<sup>-3</sup> PNA mg tissue 10<sup>-1</sup> (9). Myeloperoxidase activity (MPO) was determined by the procedure of Suzuki et al. (10). Samples were homogenized in 50 mM phosphate buffer pH 5.4 and 0.5% hexadecyltrimethylammonium bromide, incubated at 4°C for 20 min, and then centrifuged at 12,000 x g for 30 min. The supernatant (100 µl) was mixed with 0.9 ml buffer to obtain a final concentration of 0.3 mM H<sub>2</sub>O<sub>2</sub>, 1.6 mM TBM (3', 5' tetramethylbenzidine substrate), 4% dimethylformamide, and 80 mM phosphate buffer. The

changes in absorbance were measured at 655 nm and recorded during 1 min each 10 sec starting at 30 sec at 25°C. The results were expressed as Δ abs 655 mg protein<sup>-1</sup>. Collagen was determined according to the method of Walsh et al. (11) with slight modifications. Samples were hydrolyzed in 6 N HCl for 18 h at 116°C. To adjust the pH after hydrolysis, a drop of phenolphthalein was added, followed by diluted potassium hydroxide solution. Then 500 µl of 10% alanine solution, pH 7.5, and 1 ml of potassium borate buffer, pH 8.7, were added to the samples. The samples were mixed, and hydroxyproline was converted to pyrrole-2-carboxylic acid with 1 ml of 0.2 M chloramines-T solution. After 25 min, the incubation reaction was stopped by adding 1.5 ml of 3.6 M sodium thiosulfate solution. After oxidation, 2.5 ml toluene was added and the tubes were shaken vigorously. The toluene layer was removed and the aqueous layer was added to a water bath and boiled for 30 min. Hydroxyproline was determined by removing 2 ml and mixing it with 1 ml of Ehrlich's reagent, allowing 30 min for color develop. Optical density was then read at 560 nm.

Alkaline phosphatase was measured by an appropriate kit reagent (Wiener Lab, Argentina) (I: 21.6 µmol of sodium phenylphosphate buffer carbonate, pH 9.8, in 6.4 ml water; II: diazo reagent, and 4.5 mg of naphthaline 1-5 disulfonate diazonium salt (5-nitro-2 aminoanisole in 4.5 ml of distilled water). Samples (500 µl) in phosphate buffer (10 mM, pH 7.4) were incubated at 37°C with 1 ml of reagent I for 30 min. The reaction was stopped by adding 200 µl of phosphatase inhibitor (2% borate salt, 1.9% formol, 850% L-disodium, 12% ETHO). Diazo reagent was added to the samples (300 µl) and the reaction was read at 490 nm. Activity was expressed in mU mg<sup>-1</sup>.

## Results

Plasma glucose levels in control (C) and diabetic rats measured at 30 and 90 days after STZ administration were: 5.5 ± 0.53, 15.1 ± 1.00 (DI), and 33.5 ± 3.44 (DII) mM/L respectively ( $P < 0.001$ , Control vs. diabetics DI and II), and the corresponding protein concentrations were: 0.397 ± 0.031, 0.296 ± 0.021, 0.145 ± 0.011, mg/mg tissue respectively ( $P < 0.001$  vs. control).

The nitrites and kallikrein of control and diabetics rats are shown in Figs. 1 and 2. Nitrite levels were increased significantly in DI, but were markedly reduced in DII ( $P < 0.001$ ).

On the other hand, the kallikrein activity was increased in DI and markedly increased in DII ( $P < 0.001$ ). Myeloperoxidase (MPO) activity was increased in DII when compared to the control ( $P < 0.001$ ) (Fig.3). When alkaline phosphatase (AP) was determined in dental pulp

at DI and DII a significant difference was observed in comparison with the controls, ( $P < 0.001$ ) (Fig. 4). Both DI and DII rats showed a significant reduction in collagen concentration when compared to the control ( $P < 0.001$ ) (Fig.5).

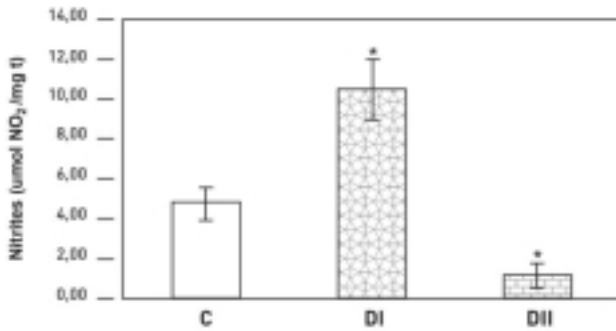


Fig. 1 Nitrite concentrations in dental pulp from control rats (C), and rats after 30 days (DI) and 90 days (DII) of diabetes. Each group represents 8-10 samples. Significant differences from control vs. DI or DII \*  $P < 0.05$

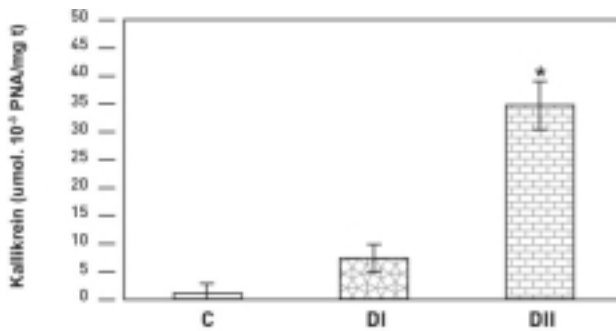


Fig. 2 Kallikrein activity in dental pulp from control rats (C), and rats after 30 days (DI) and 90 days (DII) of diabetes. Each group represents 8-10 samples. Significant differences from control vs. DI or DII \*  $P < 0.05$

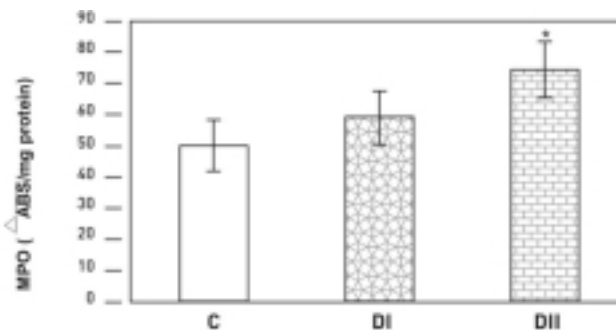


Fig. 3 Myeloperoxidase activity in dental pulp from control rats (C), and rats after 30 days (DI) and 90 days (DII) of diabetes. Each group represents 8-10 samples. Statistical differences from control and DI vs. DII \*  $P < 0.05$

## Statistical analysis

Data are expressed as mean  $\pm$  SEM. Differences between normal controls and diabetic rats were analyzed by regression analysis and unpaired Student's *t*-test. Differences at  $P < 0.05$  were considered to be statistically significant.

## Discussion

It is well established that diabetes mellitus is associated with oral complications and with depression of natural defenses against infection (12). Patients with poorly controlled diabetes have a chronic metabolism disorder of carbohydrate and lipid metabolism, and are prone to gingival (13) and dental injuries (3). Because dental pulp has limited or no collateral circulation, conditions induced by diabetes, such as periodontitis, may affect the dental pulp via periapical way (14). Our results provide evidence for two important types of dental pulp damage according to the duration of diabetes. The first one occurs after a short initial period of diabetes when hyperglycemia is around

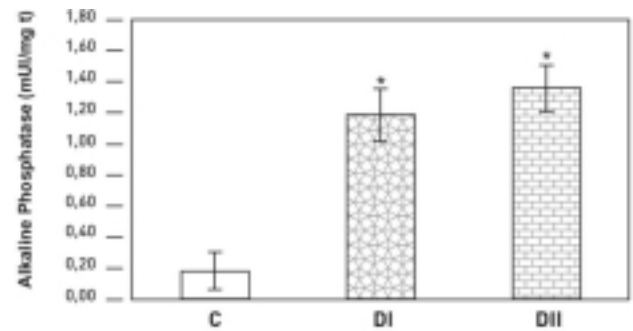


Fig. 4 Alkaline phosphatase in dental pulp from control rats (C), and rats after 30 days (DI) and 90 days (DII) of diabetes. Each group represents 8-10 samples. Statistical differences from control vs. DI and DII \*  $P < 0.05$

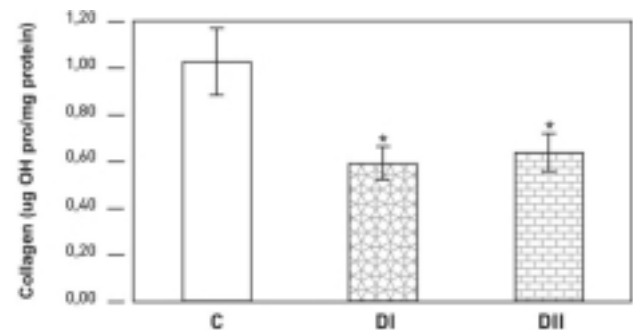


Fig. 5 Collagen concentration in dental pulp from control rats (C), and rats after 30 days (DI) and 90 days (DII) of diabetes. Each group represents 8-10 samples. Statistical differences from control vs. DI and DII \*  $P < 0.05$

15.1 mm/l and may cause early inflammatory alterations with increased concentrations of kallikrein and nitrites. The type of cells synthesizing iNOS in the early phase of pulp inflammation are neutrophil and macrophages (15). Various kinds of pro-inflammatory mediators have been demonstrated to have a possible role in the development of pulp inflammation (16). Of all the known cell mediators, the kallikrein-kinin system, is considered to play a primary role in the onset of the inflammation (17). The kallikrein-kinin system releases cytokines (IL-1, TNF) (18) and many second generation mediators (prostaglandins and leukotrienes). Elevated kallikrein enzymes levels are observed in mice with early-phase diabetes (19). Our results indicate that the initial increase of dental pulp kallikrein may occur as a consequence of the inflammatory process of diabetic hyperglycemia and the limited defense capacity of dental pulp, which is covered by hard dental tissues. Bradykinin released from neutrophil kininogen is a mediator of the early inflammatory process, especially through promotion of venule constriction and increased capillary membrane permeability (20).

Both generalized accumulation of atheromatous deposit in the vessels lumen (21) and an increased endothelial cell permeability occur (22). These changes impair the leukostatic response and decrease the leukocyte microbicidal activity (23). Myeloperoxidase activity (MPO) is well known a marker of neutrophil infiltration (24). Thus, neutrophils and macrophages appear to be responsible for the production of NO observed in the early phase of diabetic pulp inflammation.

The onset of MPO observed at 30 days after STZ treatment suggest pulp infiltration by PMNs and macrophages. Interestingly, at 30 days of diabetes the dental pulp AP was significantly increased and the collagen concentration was markedly decreased. The present results confirm that AP activity starts to increase during the initial phase of diabetes and is related to the events that characterize the different phases of diabetic inflammation. On the other hand, collagen synthesis is significantly affected when advanced glycation end-products (AGEs) tend to increase. In addition it is well known that collagenase activity in animal tissues is increased in association with diabetes (25). The second important state of dental pulp damage is a longer period of diabetes (90 days onwards), causing irreversible changes on dental pulp leading to necrosis.

Our data demonstrated progressive deterioration of the matrix components, attributable to chronic hyperglycemia and a rapid oxidation of NO to non-reactive species (26,27). Two other inflammatory components were significantly increased during the chronic phase of diabetes: kallikrein

and MPO.

The functional role of kallikrein may be specific to the diabetes process as described previously (19). In addition, MPO secreted by PMNs into the extracellular milieu catalyzes the conversion of hydrogen peroxide to hypochlorous acid as a primary product responsible for cytotoxicity of neutrophils (28). In addition, increased levels of MPO have been observed in diabetic retinopathy (29). The increase in the activity of both MPO and kallikrein enzymes in longer-standing diabetes could reflect the inflammatory process and the formation of AGEs. The formation of AGEs plays a central role in diabetic complications (30). On the other hand, the increase of AP could be related to the continuous inflammatory process of diabetes as observed in kallikrein activity. Since collagen concentrations were decreased at 90 days of diabetes, the AGE formation with chronic hyperglycemia could be a primary reason for changes in collagen stability and vascular integrity (31). Similar results have recently been found in gingiva of diabetic rats (6). In conclusion, progressive diabetic hyperglycemia in this experimental model shows modifications of dental pulp components that may be involved in tissue changes not only in the periodontium, but also at the dental pulp. It may eventually become possible to control these modifications at the early state of diabetes, and to devise strategies for the success of pulp treatment.

### Acknowledgments

This study was supported by a grant from Universidad Argentina John F. Kennedy and the Universidad de El Salvador, School of Dentistry and Medicine. English translation by Maria V. Gonzalez Eusevi, revised by Maria F. Jaca.

### References

1. Garber AJ (1998) Diabetes mellitus. In Internal medicine, Stein JH ed, Mosby, St Louis, 1850-1854
2. Stratton IM, Adler AI, Neil HAW, Matthews DR, Manley SE, Cull CA, Hadden D, Turner RC, Holman RR (2000) Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *BMJ* 321, 405-412
3. Russell BG (1967) The dental pulp in diabetes mellitus. *Acta Pathol Microbiol Scand* 70, 319-320
4. Amatyakul S, Chakraphan D, Chotpaibulpan S, Patumraj S (2003) The effect of long-term supplementation of vitamin C on pulp blood flow in streptozotocin-induced diabetic rats. *Clin Hemorheol Microcirc* 29, 313-319

5. Kohsaka T, Kumazawa M, Yamasaki M, Nakamura H (1996) Periapical lesions in rats with streptozotocin-induced diabetes. *J Endod* 22, 418-421
6. Catanzaro OL, Dziubecki D, Hanisch I, Diaz N, Martinez Ceron C, Filipo S, Rodriguez RR (2005) Las complicaciones de la diabetes: La enfermedad periodontal. *Rev Soc Argent de Diabetes* 39, 10-15 (in Spanish)
7. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193, 265-275
8. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite, and [<sup>15</sup>N] nitrate in biological fluids. *Anal Biochem* 126, 131-138
9. Vila SB, Peluffo VA, Alvarado C, Cresto JC, Zuccollo A, Catanzaro OL (1992) The kallikrein-kinin system in early state of diabetes. *Agents Actions Suppl* 38, 304-310
10. Suzuki K, Ota H, Sasagawa S, Sakatani T, Fujikura T (1983) Assay method for myeloperoxidase in human polymorphonuclear leukocytes. *Anal Biochem* 132, 345-352
11. Walsh BJ, Thornton SC, Penny R, Breit SN (1992) Microplate reader-based quantitation of collagens. *Anal Biochem* 203, 187-190
12. Bagdade JD, Nielson KL, Bulger RJ (1972) Reversible abnormalities in phagocytic function in poorly controlled diabetic patients. *Am J Med Sci* 263, 451-456
13. Seppala B, Ainamo J (1994) A site-by-site follow-up on the effect of controlled versus poorly controlled insulin-dependent diabetes mellitus. *J Clin Periodontol* 21, 161-165
14. Bissada NF, Sharawy AM (1970) Histologic study of gingival and pulpal vascular changes in human diabetics. *Egypt Dent J* 16, 283-296
15. Kawashima N, Nakano-Kawanishi H, Suzuki N, Takagi M, Suda H (2005) Effect of NOS inhibitor on cytokine and COX2 expression in rat pulpitis. *J Dent Res* 84, 762-767
16. Entman ML, Smith CW (1994) Postreperfusion inflammation: a model for reaction in injury in cardiovascular disease. *Cardiovasc Res* 28, 1301-1311
17. Bhoola KD, Figueroa CD, Worthy K (1992) Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol Rev* 44, 1-80
18. Tiffany CW, Burch RM (1989) Bradykinin stimulates tumor necrosis factor and interleukin-1 released from macrophages. *FEBS Lett* 247, 189-192
19. Zuccollo A, Navarro M, Catanzaro OL (1996) Effects of B1 and B2 kinin receptor antagonists in diabetic mice. *Can J Physiol Pharmacol* 74, 586-589
20. Bhoola KD (1996) Translocation of the neutrophil kinin moiety and changes in the regulation of kinin receptors in inflammation. *Immunopharmacology* 33, 247-256
21. Brownlee M, Cerami A, Vlassara H (1988) Advanced products of nonenzymatic glycosylation and the pathogenesis of diabetic vascular disease. *Diabetes Metab Rev* 4, 437-451
22. Listgarten MA, Ricker FH Jr, Laster L, Shapiro J, Cohen DW (1974) Vascular basement lamina thickness in the normal and inflamed gingiva of diabetics and non-diabetics. *J Periodontol* 45, 676-684
23. Hill HR, Sauls HS, Dettloff JL, Quie PG (1974) Impaired leukotactic responsiveness in patients with juvenile diabetes mellitus. *Clin Immunol Immunopathol* 2, 395-403
24. Deguchi S, Hori T, Creamer H, Gabler W (1990) Neutrophil-mediated damage to human periodontal ligament-derived fibroblasts: role of lipopolysaccharide. *J Periodontal Res* 25, 293-299
25. Golub LM, Schneir M, Ramamurthy NS (1978) Enhanced collagenase activity in diabetic rat gingiva: in vitro and in vivo evidence. *J Dent Res* 57, 520-525
26. Tesfamariam B, Brown ML, Cohen RA (1991) Elevated glucose impairs endothelium-dependent relaxation by activating protein kinase C. *J Clin Invest* 87, 1643-1648
27. Tesfamariam B, Cohen RA (1992) Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am J Physiol* 263, H321-326
28. Weiss SJ (1989) Tissue destruction by neutrophils. *N Engl J Med* 320, 365-376
29. Augustin AJ, Breipohl W, Boker T, Lutz J, Spitznas M (1993) Increased lipid peroxide levels and myeloperoxidase activity in the vitreous of patients suffering from proliferative diabetic retinopathy. *Graefes Arch Clin Exp Ophthalmol* 231, 647-650
30. Vlassara H (1997) Recent progress in advanced glycation end products and diabetic complications. *Diabetes* 46, Suppl 2, S19-25
31. Salmela PI, Oikarinen A, Pirttiaho H, Knip M, Niemi M, Ryhanen L (1989) Increased non-enzymatic glycosylation and reduced solubility of skin collagen in insulin-dependent diabetic patients. *Diabetes Res* 11, 115-120