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Long term bone alterations in aged rats suffering type 1 diabetes

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ABSTRACT

Increasing duration of type 1 diabetes mellitus alters bone metabolism. Clinical studies and experimental studies in long bones of rats with experimentally induced diabetes have reported a decrease in bone density. Few studies have explored this diabetes related alteration in the maxillae. Given that this finding could indicate the possible development of osteopenia in the maxilla in the long term, the present study sought to analyze alterations in alveolar bone in aged rats, 12, 18, and 24 weeks after inducing diabetes, and compare alveolar bone response to that of tibial subchondral bone at the same experimental times. Thirty-six male Wistar rats, 130 g body weight, were divided into 2 groups: an experimental group (E) receiving a single i.p. 60 mg/kg dose of streptozotocin, and a control group (C). Both the control and experimental groups were divided into 3 sub-sets, according to the time of euthanasia: 12, 18 and 24 weeks. The alveolar bone and tibiae were examined histologically and histomorphometrically. The results were analyzed using Student's *t*-test; a value of $p < 0.05$ was considered statistically significant. Results: Subchondral bone volume and bone activity/remodeling, mainly bone rest, were significantly lower in diabetic animals compared to controls, at both 12 and 18 weeks. No differences in alveolar bone parameters were observed between diabetic and control animals at either of the experimental times. Animals surviving at 24 weeks showed few trabeculae at rest and severe destruction of dental and periodontal tissues. The results of the present study show that diabetic osteopenia is evident in the tibia at 12 and at 18 weeks, whereas its effects on the maxilla can be seen at 24 weeks, with substantial destruction of alveolar bone and of the remaining periodontal and dental tissues. All the above observations highlight the need for preventive oral care in diabetic patients, before irreversible damage to dental and periodontal tissues occurs.

1. Introduction

Type 1 diabetes mellitus is characterized by a condition of hyperglycemia as a result of a total deficiency in insulin synthesis by spleen β cells. Long-term complications include retinopathy, nephropathy, peripheral and autonomic neuropathy, cardiovascular disease (Armas et al., 2012; Bensch et al., 2003). Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss and susceptibility to infections. Decreased salivary flow and increased exposure to bacteria as a consequence of elevated salivary glucose levels result in increased bacterial substrate, favoring caries and periodontal disease (Misawa et al., 2007; Jindal et al., 2015). Patients with poorly controlled or uncontrolled type I diabetes can present other oral disorders, such as dry mucous membranes (xerostomia), oral candidiasis, burning mouth or tongue (glossopyrosis), impaired wound healing, recurrent oral infections and acetone breath (Ship, 2003; Alves et al., 2015). Factors that might contribute to oral complications in these patients include decreased polymorphonuclear leukocyte function, abnormal collagen metabolism and prolonged wound healing time (Bensch et al., 2003). Altered protein metabolism resulting from impaired use of glucose can contribute to increased breakdown of collagen in the connective tissues. In addition, the altered protein metabolism might add to the impaired healing responses in diabetic patients (Nyman et al., 2011).

It is well known that alterations in bone metabolism of long bones are a common finding in patients with diabetes type 1 (Merlotti et al., 2010; Mamiko and Tsukamoto, 2010; Iwamoto et al., 2011; Zhao et al., 2013). Previous studies in rat tibia conducted at our laboratory showed a decrease in bone density concomitant with microstructural alterations in subchondral bone one week post-experimental induction of type I diabetes (Villarino et al., 2006), and a more marked decrease at 6 weeks (Pulitano Manisagian et al., 2014).

Nevertheless, there are few reports on the effect of diabetes on alveolar bone. Mishima et al. (2002) reported lower bone formation and bone remodeling in rats 4 weeks post-induction of diabetes, as shown by dynamic histomorphometry. Villarino et al. (2003) found that pups born to type I diabetic dams had a smaller mandible, showing lower alveolar bone volume in bone undergoing formation than healthy controls. In addition, Ubios et al. (2010) observed a greater number of sclerostin positive osteocytes in alveolar bone in diabetic rats compared to controls (Ubios et al., 2010). Sclerostin is a secreted Wnt antagonist produced almost exclusively by osteocytes that binds to the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6), inhibiting the canonical Wnt/β-catenin signaling pathway and thus

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osteoblast activity (Genari et al., 2012). However, Villarino et al. (2011) found no alterations in rat alveolar bone 6 weeks post-induction of diabetes. Given that this observation suggests the potential onset of osteopenia in the maxillae in long standing diabetes, the aim of the present work was to study alterations in alveolar bone in aged rats at 12, 18, and 24 weeks after inducing diabetes, and compare alveolar bone and tibial subchondral bone response at the same experimental times.

2. Materials and methods

Thirty-nine male Wistar rats weighing 130 g body weight were assigned to one of two groups, experimental or control. Animals in the experimental group (21) were subjected to induction of diabetes on day 1 of the experiment by intraperitoneal administration of a single 60 mg/kg body weight dose of streptozotocin dissolved in 1 mm of citrate buffer, pH 4.0, (Sigma-Aldrich, Inc. St. Louis, Mo). Control animals received an equal volume of citrate buffer under the same conditions as the experimental group.

Forty-eight hours after the onset of the experiment, blood glucose levels were determined by the glucose oxidase method, using Accu-Check Sensor Comfort test strips (Roche Diagnostics Ltd., Santiago, Chile) in an Accu-Chek Sensor (Roche Diagnostics GmbH, Mannheim, Germany). Eighteen of the 21 streptozotocin-treated rats had glucose levels higher than 250 mg/dL and were included in the experimental group; 3 did not develop hyperglycemia and were therefore excluded. Eighteen untreated animals with glucose levels below 120 mg/dL served as controls. Both groups were divided into 3 subsets of 6 animals each, and euthanized at different time points: 12, 18, and 24 weeks.

The rats were housed in groups of 3 in cages with wood chip bedding; cage bedding was changed three times per week in control cages and daily in experimental cages. All control and experimental animals were fed the same rat chow and were allowed free access to food and water. The rat chow (Brand name: "Cooperación"), has SENASA (National Agriculture and Food Quality and Health Service of Argentina) approval, N°04–288/A; and is manufactured by ACA (Argentine Food Manufacturing Cooperative). Formulation specifications: Humidity (maximum): 12%; Protein (minimum): 23%; Ethereal Extract (minimum): 5%; Crude fiber (maximum): 6%; Total Mineral (maximum): 10%; Calcium (min-max): 1–1.4%; Phosphorus (min-max) 0.5–0.8%; Chloride 0.3%; Sodium 0.2%; Potassium 0.7%; Magnesium 0.2%; Sulfur 0.16%.

Housing conditions included controlled temperature (20–26 °C) and humidity (40–70%), and standard 12 h light-dark cycles (lights on at 7:00 am; lights off at 7:00 pm).

All animal procedures were conducted in keeping with the guidelines of The National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication 85-123 Rev.2010), and were approved by the Ethics Committee of the School of Dentistry of the University of Buenos Aires (FOUBA-UBACYT 2011-2014-3).

Immediately after euthanasia, the maxilla and tibiae were resected, fixed in buffered formalin (pH 7.0) for 48 h, decalcified in 10% EDTA for 30 days, and embedded in paraffin. Buccopalatal oriented sections of the maxilla at the level of the distal roots of the first upper molar, and longitudinal frontal sections of the tibia were obtained and stained with hematoxylin-eosin for histological examination.

Histomorphometric studies of histological sections obtained at 12 and 18 weeks post-induction of diabetes were performed based on the stereological principles of Weibel and Elias (1967) and using the nomenclature established by Parfitt (1987) and revised by Dempster et al. (2013). For this purpose, digital microphotographs of the histological sections were taken using a Canon Powershot A640, 10.0 megapixel, 4× optical zoom digital camera (Canon Inc., Tokyo, Japan) mounted on a Carl Zeiss Axioskop 2 microscope (Carl Zeiss Mikroscopie, Jena, Germany), and used to measure the following histomorphometric parameters using Image Pro ® Plus software, version 5.1 (Media Cybernetics).

a. In tibia sections:
   - Subchondral bone volume (BV/TV), defined as the fraction of total volume corresponding to trabecular bone volume, measured in a 40,000 μm^2 rectangular area in the middle region below the epiphyseal cartilage; results are expressed as a percentage.
   - Bone activity determined in the same area of subchondral bone by measuring:
     - Eroded surfaces: ES/BS (%): Bone resorption surfaces, covered with active osteoclasts
     - Osteoblasts surfaces: ObS/BS (%): Bone formation surfaces, covered with active osteoblasts.
     - Lining cell surfaces: LCS/BS (%): Resting bone surfaces, covered with bone lining cells.

b. In alveolar bone sections:
   - Bone volume (BV/TV)
   - Bone activity at the surface of the periodontal cortical plate of alveolar bone.

Sections obtained at 24 weeks were examined histologically, but were not studied histomorphometrically in the present work.

2.1. Statistical analysis: the results are expressed as mean and standard deviation

The data were statistically analyzed by Student's *t*-Test, using "Primer of Biostatistics" software. Statistical significance was set at a value of $p < 0.05$.

3. Results

The histologic study showed tibial subchondral bone of diabetic animals to have fewer and thinner bone trabeculae than controls, and more markedly so at 18 weeks than at 12 weeks. Larger areas of bone lining cells on bone trabeculae were observed in experimental sections than in controls. These observations are consistent with the

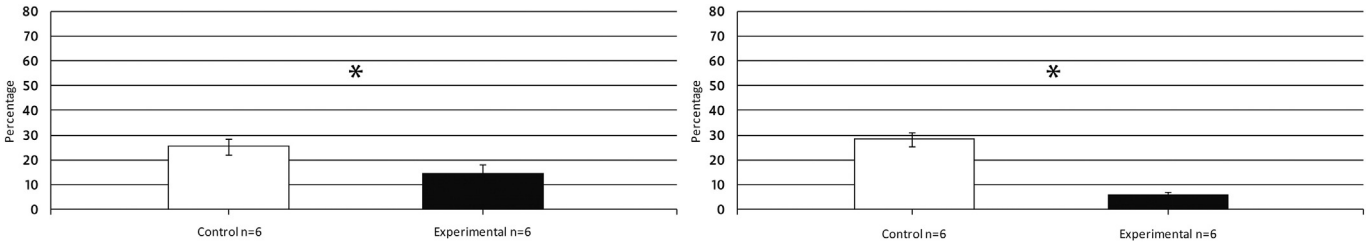

Fig. 1. Histomorphometric study of subchondral BV/TV. Left: at 12 weeks. Right: at 18 weeks. Significantly lower BV/TV was observed in diabetic animals compared to controls at both experimental times * ($p < 0.05$).

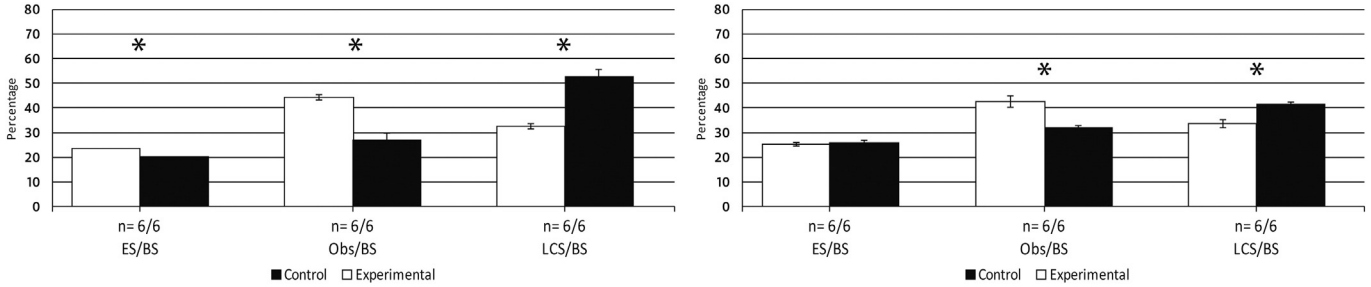

Fig. 2. Histomorphometric study of bone activity in bone trabeculae in tibial subchondral bone. Left: at 12 weeks. Right: at 18 weeks. A higher percentage of resting surfaces and lower percentage of formation surfaces were observed in diabetic animals compared to controls, at both experimental times. The differences between groups were statistically significant * ($p < 0.05$).

histomorphometric results showing lower bone volume (12 weeks: C: 24.79% ± 4, E: 14.4% ± 3.86 $p < 0.005$; 18 weeks: C: 27.8% ± 3.08, E: 5.64% ± 1.08 $p < 0.005$) (Fig. 1) as well as a significantly higher percentage of resting surfaces (12 weeks: C: 32.42% ± 1.33, E: 52.91% ± 2.97 $p < 0.05$. 18 weeks: C: 33,9% ± 2.56, E: 41.88% ± 1.27 $p < 0.05$) and a significantly lower percentage of formation surfaces in diabetic animals than in controls (12 weeks: C: 44.13% ± 1.44, E: 26.89% ± 2.92 $p < 0.05$; 18 weeks: C: 42.47% ± 3.83, E: 32.05% ± 0.97 $p < 0.05$). No significant differences in bone resorption surfaces were observed between groups at any of the studied time points (12 weeks: C: 23.42% ± 0.65, E: 20.18% ± 0.6. 18 weeks: C: 25.28% ± 1.32, E: 26.05% ± 1.63) (Fig. 2).

The histomorphometric study of alveolar bone volume showed no statistically significant differences between the control and experimental groups at either 12 or 18 weeks (12 weeks: C: 30.89% ± 1.81, E: 27.83% ± 1.44. 18 weeks C: 40.46% ± 4.95, E: 45.63% ± 6.64) (Fig. 3).

The periodontal cortex of diabetic animals was found to have more bone resting surfaces than controls. This finding was consistent with histomorphometric observations at both experimental times. In addition, experimental sections exhibited more bone resorption surfaces than controls at 12 weeks, though not at 18 weeks. (12 weeks: Resorption: C: 19.16% ± 8.33, E: 26.02% ± 9.27; Formation: C: 30.13% ± 5.49, E: 22.59% ± 6.94; Rest: C: 50.18% ± 6.77, E: 53.19% ± 4.77. 18 weeks: Resorption: C: 22.06% ± 9.44, E: 21.77% ± 7.44; Formation: C: 24.22% ± 8.9, E: 18.73% ± 10.14; Rest: C: 61.71% ± 11.78, E: 56.95% ± 3.7.) (Fig. 4).

Given that 4 of the 6 animals in the third experimental group died between week 18 and week 24, the two surviving animals were used for descriptive analysis only. At this time point, tibial subchondral bone of diabetic animals showed thinner epiphyseal cartilage and fewer and thinner bone trabeculae as compared to controls. In addition, serious destruction of alveolar bone as well as marked deterioration of dental and periodontal tissues were observed in both surviving rats. Dental tissue damage included coronal substance loss, beaded dentinal tubules, and disorganized pulp tissue with a necrotic appearance. At the periodontal level, experimental sections showed chronic infiltrate in the furcation area, periapical abscesses, and fewer and thinner trabeculae in alveolar bone, as compared to controls.

4. Discussion

Histologically, the tibiae and maxillary alveolar bone of diabetic animals at both 12 and 18 weeks featured scant bone formation surfaces with a strong predominance of resting surfaces, which is compatible with osteopenia. The osteopenic condition of the tibia and serious destruction of periodontal tissues and alveolar bone were more evident in animals surviving a 24 weeks.

A number of clinical and experimental studies have reported less bone formation in type 1 diabetes (McCabe, 2007; Merlotti et al., 2010), as shown by serologic (Iitzuka et al., 2013) and densitometric (Abd El Dayem et al., 2011) studies.

Experimental histomorphometric studies showed osteopenia in rat tibiae 12 weeks after inducing diabetes (Silva et al., 2009), and lower bone remodeling in the tibiae of mice (Hamada et al., 2007). The same results were observed in the femurs of mice 18 weeks post-induction mouse femur (Nyman et al., 2011). Our observations in the tibia are in line with the aforementioned reports. With regard to alveolar bone, a study by Mishima et al. (2002) using static histomorphometry showed a decrease in bone turnover rates 10 weeks after inducing diabetes. Our results showed that though bone activity values in alveolar bone were lower than controls at 12 and 18 weeks, the difference did not reach statistical significance. This finding is in line with observations by Kim et al. (2014) in diabetic rats 4 weeks post-induction. It must be pointed out that no studies reported to date performed histological studies as late as 24 weeks after inducing diabetes.

The difference in the behavior of both bones may be associated with their different embryologic origin. Alveolar bone, which is part of the attachment complex of the periodontium, arises from neural crest mesenchyme, whereas the tibia arises from the mesoderm. This fact was pointed out by Mavropoulus et al., 2007, when the authors observed different response of these two bones to estrogen-deprivation in female rats. We suggest that other local factors, such as post-eruption movements and mechanical stress of mastication, are also associated with the differences in response.

There is certain controversy in the literature as to whether diabetic osteopenia is associated with an alteration in osteoclastogenesis and

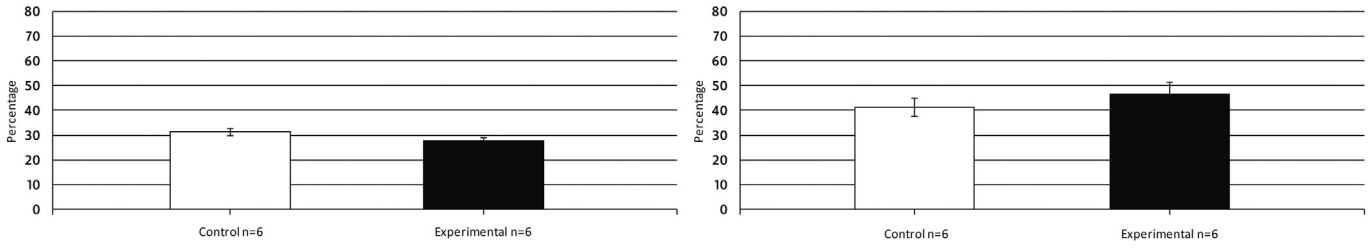

Fig. 3. Histomorphometric study of BV/TV in interradicular bone. Left: at 12 weeks. Right: at 18 weeks. No differences were observed between diabetic and control animals at either of the experimental times.

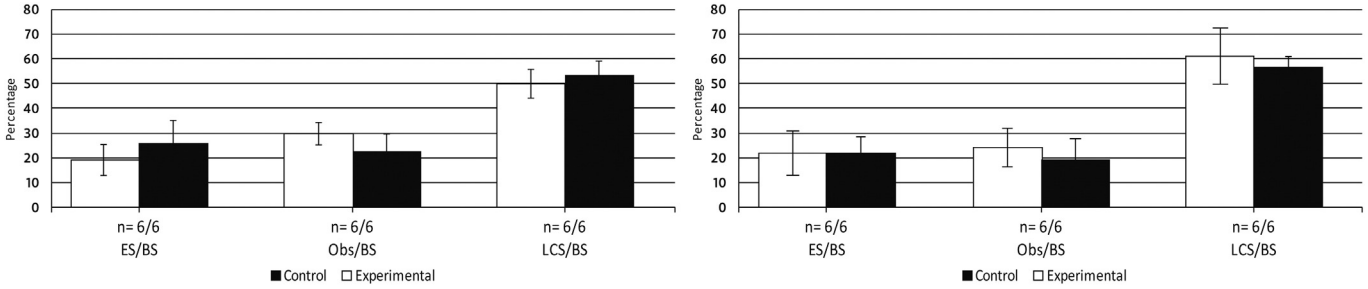

Fig. 4. Histomorphometric study of bone activity in the periodontal plate of alveolar bone. Left: at 12 weeks. Right: at 18 weeks. A higher percentage of resting surfaces was observed in diabetic animals compared to controls at both experimental times.

bone resorption, or to a depression in bone formation. In line with observations reported by McCabe (2007) and Merlotti et al. (2010), Hamada et al. (2009) found a decrease in bone formation and resorption in Type1 diabetic rats 12-weeks post-induction of diabetes.

The histomorphometric results obtained at the time points studied here showed higher bone resorption in experimental animals than in controls at 18 weeks; nevertheless, the difference between groups was not statistically significant. The two animals surviving at 24 weeks showed severe destruction of alveolar bone together with severe deterioration of dental and periodontal tissues, similar to the dental problems observed in diabetic patients with poor oral care. In the present study, the dental and periodontal deterioration was found to occur between weeks 18 and 24 post-induction of diabetes. Although the very small sample size at 24 weeks is a limitation to the present study, it could be posited that the observed deterioration may be associated with the longstanding diabetes. There are no reports in the literature assessing the effect of diabetes on dental and periodontal tissues, using such a long experimental time as the time point used here, i.e. 24 weeks. Therefore, further longitudinal experimental studies using a larger sample size to allow for statistical analysis of the results, and performing measures at time points between 18 and 24 weeks, would contribute to understanding the progression of dental damage that occurs in type 1 diabetes.

The results of the present study showed that tibial subchondral bone of diabetic rats had lower bone volume and fewer and thinner bone trabeculae as compared to controls; these alterations were more pronounced at 18 weeks than at 12 weeks. In addition, the severe dental and periodontal alterations observed here highlight the importance of preventive oral care for diabetic patients from the moment diabetes is detected, before irreversible damage to dental and periodontal tissues occurs.

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