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Effects on Alveolar Bone of Diabetes Mellitus Induced by Streptozotocin in Rats. Histopathologic and Immunohistochemical Study

Efectos de la Diabetes Mellitus Inducida por Streptozotocina en el Hueso Alveolar de Ratas. Estudio Histopatológico e Inmunohistoquímico

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SUMMARY: The purpose of this study was to investigate effects of diabetes mellitus (DM) on the alveolar bone with histopathological and immunohistochemical methods. Wistar rats were divided into two groups, control and diabetes group. Control group was fed standard rat chow and drinking water for 8 weeks. Single dose (Streptozotocin) STZ (55 mg/kg), was dissolved in sodium citrate buffer and introduced intraperitoneal injection. Diabetes group and control group were compared in terms of glucose values. The blood glucose concentration in diabetic rats was significantly high ($p < 0.05$). In diabetes group; periodontal membrane and the dilation of blood vessels, hemorrhage has also been a significant increase in inflammatory cells. In the diabetes group, osteonectin showed positive expression in periodontal membrane and showed negative expression in osteocytes of alveolar bone. Osteopontin expression in fibroblast cells and periodontal membrane collagen fibrils was positive, alveolar cells, osteocytes and bone matrix bone was found positive. Diabetes results showed that there formed periodontitis; due to the increase in inflammation inhibiting bone formation delaying the development of early bone cells.

KEY WORDS: Alveolar bone; Diabetes mellitus; Rat; Osteonectin; Osteopontin.

INTRODUCTION

Diabetes mellitus is a disease in which carbohydrate, protein and lipid metabolism homeostasis are inadequately regulated by the pancreatic hormone, insulin, resulting in an increase in blood glucose levels (Sunil *et al*., 2011). Type 2 diabetes mellitus (T2DM) is a multiple risk factor disease, and dyslipidemia, hyperglycemia and visceral obesity are the major determinants (Preshaw *et al*., 2012). Periodontal diseases are the six most common co-morbid condition in patients with diabetes mellitus (Löe, 1993; Al-Maskari *et al*., 2011) and evidence indicates a bidirectional relationship between these two pathologies (Lakschevitz *et al*., 2011; Awuti *et al*., 2012).The presence of diabetes influences the periodontal tissues by altering neutrophil function and collagen synthesis, inducing vascular abnormalities through genetic predisposition (Oliver & Tervonen, 1994). Relationship between diabetes and periodontitis is thought to be due to changes occurring in the alveolar bone structure. Osteopontin (OPN) is considered to play important roles in promoting or regulating the adhesion, attachment, and spreading of osteoclasts to bone surfaces during bone resorption (Reinholt *et al*., 1990; Ikeda *et al*., 1992). OPN blood levels in diabetic patients are known to be higher than

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in normal individuals (Yan *et al*., 2010). (Adiponectin) APN is an important inflammatory molecule which modulates insulin resistance associated with a response (Ninomiya *et al*., 2007). Osteonectin is a 32 kDa phosphorylated glycoprotein that binds simultaneously to type I collagen and hydroxyapatite (Barrett-Connor & Holbrook, 1992), and is localized at osteoblasts and odontoblasts (Hamann *et al*., 2012). In this study that we aim to investigate the effects of streptozotocin-induced diabetes mellitus of histopathological and immunohistochemical changes on alveolar bone in rats.

MATERIAL AND METHOD

The present work was conducted in accordance with the guidelines for the Care and Use of Laboratory Animals from the Dicle University. The study was conducted as per approval of the Animal Experiments Local Ethics Committee, Dicle University. Experimental Animal Research Center 20 adult male Wistar rats were randomly divided into 2 groups.

Control group (n=10) was fed standard rat chow and drinking water for 8 weeks. Diabetic Control (DC) group (n=10):Single dose STZ (55 mg/kg), was dissolved in sodium citrate buffer (0.1 M,PH 4.5) and carried out intraperitoneal injection (Reinholt *et al*.). The experiment subjects fasted for 12 hours, then started to be fed with standard rat chow and drinking water after 4 hours. After 2 days, 12 hours of fasting after the application from the tail end with the capillary blood glucose meter (Contour TS Bayer) hand blood glucose levels were measured and the value 250 mg / dl or above was taken to diabetic group. At the end of the experimental period, rats were anesthetized under ketamine+xylazin and sacrificed by cardiac puncture. Blood samples were taken for biochemical tests of rats. The animals were sacrificed by decapitation. First, the maxillary regions were dissected under ketamine hydrochloride anesthesia. The samples were fixed with neutral buffered formalin solution and decalcified with 5 % ethylene-diamine tetra acetic acid. After preservation, alveolar bone samples were directly dehydrated in a graded series of ethanol and embedded in paraffin wax. Next, 4–6 µm sections were cut with a microtome (Rotatory Microtome, Leica, RM 2265, Germany) and mounted on coated slides. The sections were stained with Haematoxylin and Eosin for observation by light microscopy (Nikon Eclipse 80i).

Immunohistochemical staining. An antigen-retrieval process was performed in citrate buffer solution (pH 6.0) two times: First for 7 min, and then for 5 min in a microwave oven at 700 W. They were allowed to cool to room temperature for 30 min and washed in distilled water for 5 min twice. Endogenous peroxidase activity was blocked in 0.1 % hydrogen peroxide for 15 min. Ultra V block (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 10 min prior to the application of the primary antibodies (osteonectin antibody, mouse monoclonal, 1/200, Santa Cruz Biotechnology, or osteopontin antibody (mouse monoclonal, 1/200, Santa Cruz Biotechnology) overnight. The secondary antibody (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 20 minutes. Then the slides were exposed to streptavidin-peroxidase for 20 min. Diaminobenzidine (DAB, Invitrogen, Carlsbad) was used as a chromogen. Control slides were prepared with same procedure but no primary antibodies. After counterstaining with Trichrom-Masson stain, slides washed in tap water for 5 min, and in distilled water for 2×5 min, mounted.

Western Blotting

Cell lysis and protein quantification. The snap frozen bone was grinded to a fine powder in a chilled mortar in the presence of liquid nitrogen. Immediately after grinding, 50 mg bone powder was transferred into a microcentrifuge tube containing 250 µl RIPA lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1 % (v/v) Triton X-100, 1 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 0.2 % (w/v) sodium fluoride, 0.2 % (w/v) sodium orthovanadate and 1× protease inhibitor mixture [CompleteTM EDTA free; Roche Diagnostics] and incubated in ice for 1 h. After incubation, aliquots were snap-frozen in liquid nitrogen and stored -86 °C. All these steps were performed on ice to minimize protein degradation. Total cellular protein concentration was determined in triplicate using a BCA protein assay according to manufacturer's instructions (Pierce, Thermo scientific). The BCA assay was performed in a 96-well plate using MultiscanTM GO microplate from Thermo Scientific.

SDS-PAGE. All protein samples were resolved by 10 % (v/v) polyacrylamide gel using a Mini Protean Tetra Cell apparatus system (Bio-Rad). The protein samples were prepared in 1× SDS loading buffer (2 % (w/v) SDS, 5 % (v/v) glycerol, 0.01 % (w/v) bromophenol blue, 8 % (w/v) DTT, which was added just before heating) and heated at 100 °C for 5 min. The protein samples (20 µg) were then loaded on to the 10 % (v/v) polyacrylamide gel and electrophoresed at 200 V for 1 h in a SDS running buffer (2.4 mM Tris, 19.2 mM glycine, 0.01 % (w/v) SDS).

Membrane transfer and antibody staining. Separated proteins from the SDS-PAGE were transferred onto the PVDF membrane at 100 V for 1 h in transfer buffer (25 mM Tris, 192 mM glycine, 20 % (v/v) methanol, pH 8.3) using a Criterion Blotter Transfer System (Bio Rad). The membrane

was then blocked with 5 % (w/v) skim milk powder in PBS-T (PBS+ 0.1 % (v/v) Tween-20) for 1 h at room temperature. After blocking, the membrane was probed with primary antibodies (anti-osteopontin 1:200 dilution from Santa Cruz, anti-osteonectin 1:200 dilution from Santa Cruz and ant-b-actin 1:1000 from Abcam) for 2 h at room temperature. The membrane was then washed four times over 30 min with PBS-T before probing with horseradish peroxidase-conjugated secondary antibodies in 1:10000 dilutions for 1 h at room temperature. The membrane was again washed four times over 30 min with PBS-T. The protein bands were visualized using ECL (Bio-Rad) according to manufacturer's instruction. The images were taken using ChemiDocTM MP (Bio-Rad).

RESULTS

The serum glucose levels were found statistically significant between the two groups (Table I). The blood glucose levels of diabetic group showed significantly higher compared to control group. The blood glucose concentration in diabetic rats was significantly increased (p<0,0001).

Diabetes group; periodontal membrane and the dilation of blood vessels, hemorrhage has also been a significant increase in inflammatory cells. Hyperplasia in osteoblast cells was seen leaving collagen fibers alveolar bone in the proximal portion of the periodontal membrane. It was observed lacunar structure expansion where cells osteocytes. In the diabetes group, osteonectin showed positive expression in periodontal membrane and showed negative expression in osteocytes of alveolar bone. Osteopontin expression in fibroblast cells and periodontal membrane collagen fibrils was positive, and in alveolar cells, osteocytes and bone matrix bone was found positive.

DISCUSSION

Bone loss is one of the complications in diabetes. It has been proposed that the incidence of bone loss is different between Type 1 DM and Type 2DM (Pacios *et al*., 2012; Sealand *et al*., 2013). The worsening of alveolar bone loss observed in diabetic animals with periodontal disease supporting previous studies, since there is a bidirectional relationship between these diseases (Breivik *et al*., 2005; Kim *et al*., 2012). In a research C57BL Db/db mice that developed diabetes at six to eight weeks of age to study decreased osteoclastogenesis in bacteria-stimulated bone loss (He *et al*., 2004). Jiang *et al*. (2013) reported resorption values of alveolar bone in the upper second molar of four groups of rats at different time points. The rats with periodontal disease (PD) and diabetes mellitus (DM) showed more alveolar bone loss than those with PD alone, DM alone, and control rats at the same time point. The impact of diabetes on bone resorption has yielded contradictory findings, with some studies indicating increased osteoclast activity

Table I. Glucose levels of diabetic control group.

Groups Average of blood glucose	Mean	Standard Deviation	Difference	Test statistic	
Non-diabetic group	97,2857	5,3452	306,8572	32,032	**p<0,0001**
Diabetic group	404,1429	29,8185			

Table II. Comparison of histopathological features between the groups.

			Bone degeneration					
			nochange	weak	medium	fair	high	Total
Groups	Control group	Count	7	3	0	0	0	10
		% within Groups	70,0%	30,0%	0%	0%	0%	100,0%
	Diabetes group	Count	0	0	4	5	1	10
		% within Groups	0%	0%	40,0%	50,0%	10,0%	100,0%
Total		Count	7	3	4	5	1	20
		% within Groups	35,0%	15,0%	20,0%	25,0%	5,0%	100,0%

Chi-Square=20,00 ; p=0,0005

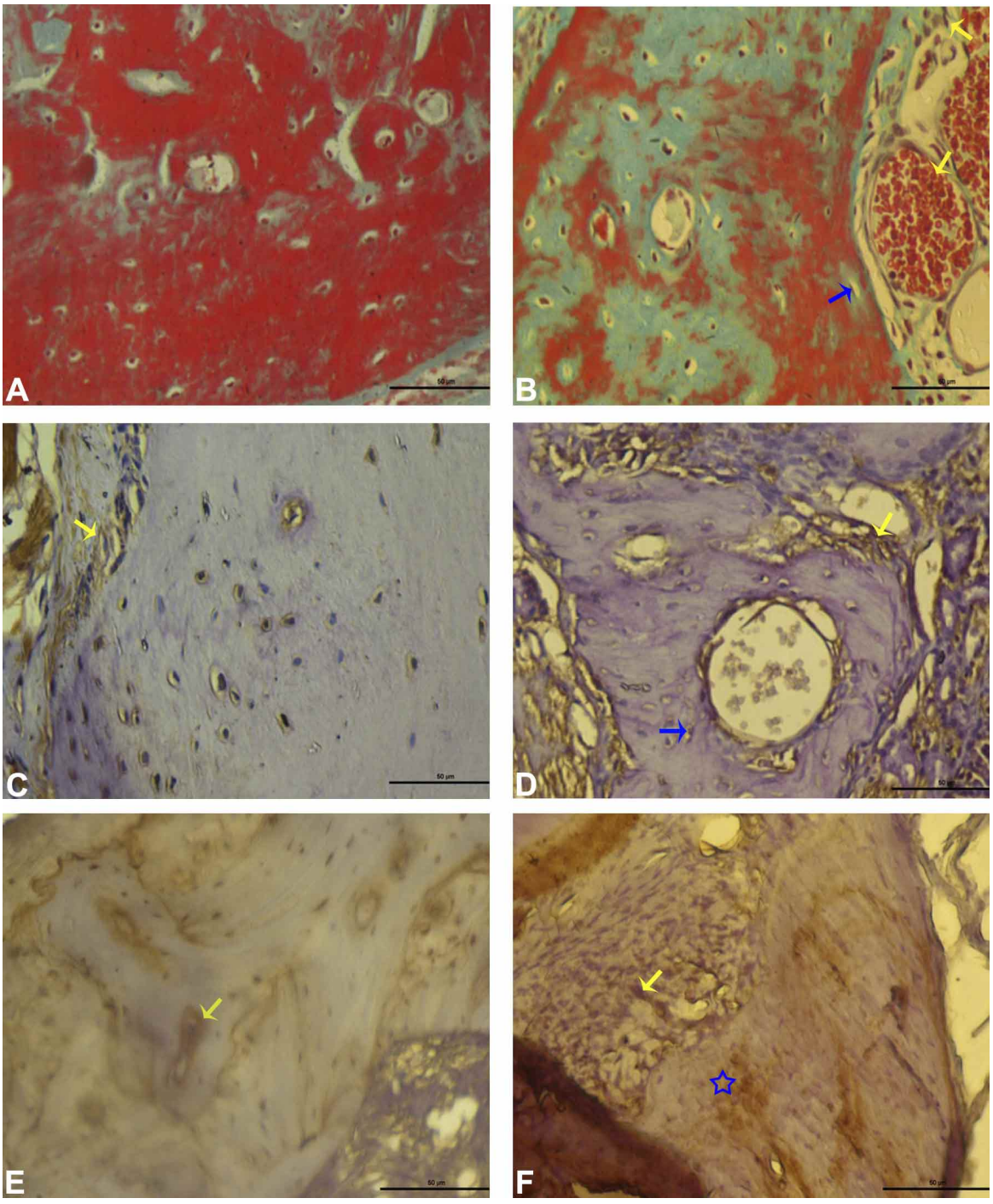

Fig. 1. A- In the control group: Regular collagen fibers appearance and blood vessels in periodontal membrane, normal distribution of osteocytes in alveolar bone Trichrom-Masson staining Bar 50 µm, B- Diabetes group: Dilatation and hemorrhage in blood vessels and an increase in inflammatory cells of periodontal membrane (yellow arrow). Hyperplasia in osteoblast cells (arrow) separating the collagen fibers in the alveolar bone sections near the periodontal membrane, lacunar structure to expand in osteocytes cells, Trichrom-Masson staining Bar 50 µm CD. C- Control group: Weak expression of osteonectin in fibroblast cells and collagen fibers (yellow arrow), of periodontal membrane Osteonectin immun-staining Bar 50 µm, D- Diabetes group: positive expression of osteonectin in periodontal membrane (yellow arrow), negative expression of osteonectin in osteocyte cells of alveolar bone (blue arrow). Osteonectin immun-staining Bar 50 µm, E- Control group: Positive expression of osteopontin in the alveolar bone matrix and haversian channels (arrow). Osteopontin immun-staining Bar 50 µm, F- Positive expression of osteopontin in bone matrix (star) and in osteocytes (yellow arrow). Positive osteopontin expression in collagen fibers and fibroblast cells of periodontal membrane (yellow arrow), Osteopontin immun-staining Bar 50 µm

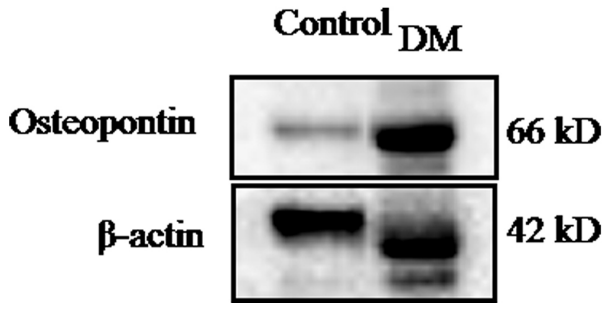

Fig. 2. The expression of osteopontin on bone was dramatically increased in Diabetes Mellitus (DM) Equal amounts of total proteins were run on the gel and analyzed by Western Blotting using anti-osteopontin and anti-b-actin antibodies. b-actin was used as a loading control.

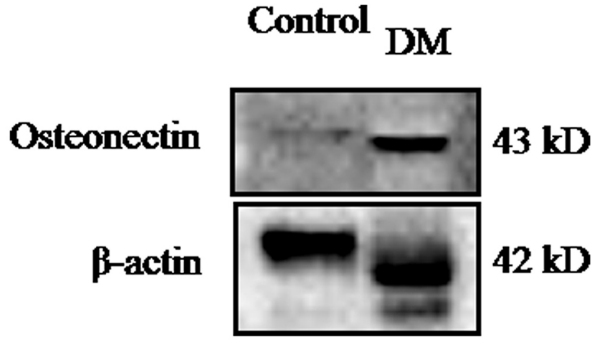

Fig. 3. The expression of osteonectin on bone was dramatically increased in Diabetes Mellitus (DM) Equal amounts of total proteins were run on the gel and analysed by Western Blotting using anti-osteonectin and anti-b-actin antibodies. b-actin was used as a loading control.

under perturbation (Gerdhem *et al*., 2005; Lozano *et al*., 2009). Diabetes has a significant effect on osteoblasts. It has consistently been noted that diabetes causes a reduction in the number of bone-forming cells (Alikhani *et al*., 2007). One of the mechanisms through which diabetes affects osteoblasts is elevated apoptosis. For example, AGEs induce osteoblast apoptosis through the MAP kinase pathway (Liu *et al*., 2006). Diabetes also increases the loss of periodontal ligament (PDL) cells that is induced by periodontal infection by accelerating apoptosis (Isaka *et al*., 2001). One important aspect of osteonectin in the context of the PDL is the potential for osteonectin to enhance collagen deposition and regeneration of diseased tissue. Periodontal disease is difficult to treat because of the associated bone loss that occurs when the PDL is degraded. Osteonectin production in bones is substantial and is also expressed in cementum, the outer layer of the tooth connecting to the PDL (Delany & Hankenson, 2009; Zuo *et al*., 2014). In our study, the periodontal ligament fibers increased collagen in the diabetic group, the hemorrhage was seen as important in inflammation and vascular changes. In the diabetic group compared to the control group, collagen fibers and inflammatory cells in osteonectin increase has led to a change of periodontal structures. However, affect the alveolar bone is thought to induce osteoblast activity in bone loss. In studies of diabetic rats hyperglycaemia has been reported to cause renal OPN production by p38 MAPK pathway (Yan *et al*.), the patient's blood, diabetes another clinical trial the amount of OPN significantly higher reported (Karamizadeh *et al*., 2013). Hyperglycemia, working directly with the induction of OPN production of this series, respectively, to support OPN hyperglycemia triggering factors of production. In this study, the expression of osteopontin in the diabetic group showed a significant increase compared to the control group. The increased expression of inflammatory cells in periodontitis is decisive support in previous studies. Periodontitis caused due to the effect of the diabetes, has increased in cell capable of OPN and migration of inflammatory cells. OPN protein is considered as a marker to determine the severity of periodontitis. Diabetes results showed that periodontitis depending on the increase in inflammation inhibits bone formation delaying the development of early bone cell.

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RESUMEN: El objetivo de este estudio fue investigar los efectos de la diabetes mellitus (DM) sobre el hueso alveolar con métodos histopatológicos e inmunohistoquímicos. Las ratas Wistar se dividieron en dos grupos, grupo control y grupo de diabetes. El grupo control fue alimentado con comida estándar y agua potable durante 8 semanas. La dosis única Streptozotocina (STZ) (55 mg/kg), se disolvió en tampón de citrato de sodio y se introdujo mediante inyección intraperitoneal. El grupo diabetes y el grupo control se compararon en términos de valores de glucosa. La concentración de glucosa en sangre en ratas diabéticas fue significativamente alta ($p < 0,05$). En el grupo diabetes hubo un aumento significativo de la membrana periodontal y dilatación de los vasos sanguíneos y hemorragia, con un aumento significativo de células inflamatorias. En el grupo diabetes, la osteonectina mostró una expresión positiva en la membrana periodontal además se observó expresión negativa en los osteocitos del hueso alveolar. La expresión de osteopontina en fibroblastos y fibrillas de colágeno en membrana periodontal fue positiva, las células alveolares, osteocitos y hueso de la matriz ósea dio positivo. Los resultados de la diabetes mostraron que existía periodontitis, debido al aumento de la inflamación que inhibió la formación ósea retardando el desarrollo de células óseas tempranas.

PALABRAS CLAVE: Hueso alveolar; Diabetes mellitus; Rata; Osteonectina; Osteopontina.

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