INFLUENCE OF SHORT-TERM DIABETES ON OSTEOCYTIC LACUNAE OF ALVEOLAR BONE. A HISTOMORPHOMETRIC STUDY

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ABSTRACT

The aim of this study was to evaluate, for the first time, the histomorphometry of the cellular and lacunar features of the osteocytes of alveolar bone in acute streptozotocin-induced diabetic insulin-treated or untreated rats . Eighteen male Wistar rats weighing 200 to 260 g were assigned to one of the following groups: I) control group (C), II) diabetic group (DBT), and III) insulin treated diabetic group (DBT+INS). Experimental diabetes was induced by a single intraperitoneal injection of 60 mg/kg of body weight of streptozotocin. Insulin treatment began 24 h after the streptozotocin injection in animals of group DBT+INS in a dose of 4-6 IU of Humulin NPH insulin given as a single daily subcutaneous (s.c.) injection each morning between 07.00 and 10.00 h. The animals were euthanized on the 8th day. The upper maxillae were removed and fixed in buffered formalin, decalcified in EDTA, embedded in paraffin and stained with H-E for histologic and histomorphometric evaluation. Bone activity and lacunar density, osteocyte and empty lacunar densities, lacunar volume and lacunar shape were evaluated. Differences between variables were assessed by one-way ANOVA. Surface bone activity values revealed that bone resorption was significantly greater in the DBT group than in the C group ($p<0.05$). Total lacunar density was significantly reduced in the DBT and DBT+INS groups as compared to control ($p<0.05$). Concomitantly, a statistically significant reduction in osteocyte density and an increase, albeit not statistically significant, in empty lacunar density was observed in DBT and DBT+INS groups versus control. Lacunar volume did not exhibit statistically significant differences. The osteocyte lacunae in the DBT group lost their rounded shape and acquired intermediate shapes. This study reveals an early response of osteocytes to hyperglycemia, before systemic compensatory mechanisms are turned on. The effects are not always compensated by insulin treatment.

Key words: *acute diabetes, osteocyte, lacunar density, hyperglycemia.*

INFLUENCIA DE LA DIABETES AGUDA EN LAS LAGUNAS OSTEOCÍTICAS DEL HUESO ALVEOLAR. ESTUDIO HISTOMORFOMÉTRICO

RESUMEN

El objetivo de este trabajo fue estudiar por primera vez las características histomorfométricas de los osteocitos y sus lagunas en el hueso alveolar de ratas con diabetes experimental aguda tratadas o no con insulina. Dieciocho ratas Wistar macho con pesos entre 200 y 260 gr fueron asignadas a uno de los siguientes grupos: I) grupo control (C), II) grupo diabético (DBT), y III) grupo diabético tratado con insulina (DBT+INS). La diabetes experimental se indujo mediante una única inyección intraperitoneal de 60 mg/kg de peso corporal de estreptozotocina. El tratamiento insulínico empezó 24 hs después de la inyección de la estreptozotocina en los animales del grupo DBT+INS en una dosis de 4-6 UI de insulina NPH Humulin, administrada diariamente por vía subcutánea cada mañana entre las 07.00 y 10.00 hs. Los animales fueron sacrificados al 8º día. Se tomaron los maxilares superiores, los cuales fueron fijados en formol buffer, descalcificados en EDTA, incluidos en parafina y coloreados con H-E para su estudio histológico e histomorfométrico. Se evaluaron la actividad ósea, las densidades lacunar, osteocitaria y de lagunas vacías, el volumen lacunar y la forma lacunar. Las diferencias entre variables fueron analizadas mediante el test de ANOVA de una vía. La actividad ósea sobre la superficie mostró que en el grupo DBT la reabsorción ósea aumentó significativamente con respecto al grupo C ($p<0.05$). La densidad lacunar total disminuyó significativamente en los grupos DBT y DBT+INS con respecto al grupo C ($p<0.05$) acompañado de una disminución estadísticamente significativa en la densidad osteocitaria y un aumento, aunque no significativo, de la densidad de lagunas vacías de los grupos DBT y DBT+INS con respecto al grupo C. El volumen lacunar no mostró diferencias estadísticamente significativas y en el grupo DBT las lagunas osteocitarias pierden su forma redondeada hacia formas intermedias. Este estudio demuestra que el osteocito responde en forma temprana a la hiperglucemia antes de que mecanismos sistémicos compensatorios se enciendan, y los cambios sufridos no siempre son compensados con el tratamiento insulínico.

Palabras clave: *diabetes aguda, osteocito, densidad lacunar, hiperglucemia.*

INTRODUCTION

Osteocytes are cells of the osteoblastic lineage that reside in lacunae within the mineralized bone matrix. They form a syncitial and communicative network between neighbouring osteocytes and cells on the bone surface through their cytoplasmic processes and canaliculi (1,2). In addition to their traditional role in the maintenance of the perilacunar matrix (3), it has been suggested that osteocyte's role in bone is associated with the modulation of osteoblast and osteoclast activity in bone remodeling and turnover (2,4-6). It has been described that bone remodeling can be modified in different conditions. Within this context, the disorders in bone remodeling that occur in type I diabetes lead to osteopenia as a chronic complication of the illness (7-11). Among other receptors, osteoblasts have receptors for insulin, and it has been shown that IGF-1 stimulates replication of preosteoblastic cells and the functional differentiation of osteoblasts *in vitro* (12). Therefore, the deficiencies in insulin production and/or action in diabetic patients reduce the action or recruitment of osteoblasts (13-15). Osteoblasts are the precursors of osteocytes. Thus, insulin disorders would also affect osteocytes, altering their morphology and viability and leading to alterations in bone turnover that in turn induce the characteristic osteopenia of the chronic diabetic state. In different metabolic bone disorders such as osteoporosis, the integrity and three-dimensional organization of the bone cell network is altered, mainly as the result of deficiencies in the connectivity of the network of canaliculi (5). In particular, this mechanism has been extensively studied in osteoporotic bones under estrogen deficiency (16,17) and glucocorticoid excess (18,19). These studies evidenced an increase in the number of osteocytes undergoing apoptosis. There is virtually no information available on the potential changes of osteocytes in diabetes. A recent *in vivo* study (20) on the ultrastructural features of the femur and tibia of streptozotocin-induced diabetic rats reported changes at 5 weeks, mainly observable in osteocytes. They showed shortening of cell processes, vacuolar formations in the cytoplasm and enlargement of the unmineralized matrix. Hormonal (5,16-19) and physical (1-5,21,22) stimuli induce alterations in the osteocyte that can be detected as changes in the lacunae and environment of osteocytes. Thus the metabolic alterations induced by diabetes might be expected to induce changes in the osteocytes. Within this context, the aim of this study was to evaluate, for the first time, the histomorphometry of the cellular and lacunar features of the osteocytes of alveolar bone in acute streptozotocin-induced diabetic insulin-treated or untreated rats.

MATERIALS AND METHODS

Eighteen male Wistar rats weighing 200 to 260 g were assigned to one of the following groups: I) control group (C), II) diabetic group (DBT), and III) insulin treated diabetic group (DBT+INS). All the animals were housed in metal cages holding no more than 6 rats each, at 21 to 24ºC and 56% humidity, under 12 hour light dark cycles and were allowed free access to water and laboratory food during the experiment. Body weight of control and experimental animals was recorded regularly throughout the study. The National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication 85-23 Rev. 1985) were observed.

Induction of experimental diabetes

Experimental diabetes was induced by a single intraperitoneal injection of 60 mg/kg of body weight of streptozotocin (SIGMA-ALDRICH, Inc., Saint Louis, USA), dissolved in freshly prepared citrate buffer (pH 4.0). Control animals received an equivalent volume of citrate buffer. Twenty-four hours after the administration of the drug, blood glucose concentrations were measured in all the animals by the glucose oxidase method with Accu-Chek Sensor Comfort test strips (Productos Roche Ltda., Santiago, Chile) in the Accu-Chek Sensor (Roche Diagnostics GmbH, Mannheim, Germany). Only rats with blood glucose concentration above 300 mg/dl after streptozotocin injection were included in the experimental groups. Normoglycemia (90-120 mg/dl) was confirmed in control animals. Streptozotocin administered as described induces an experimental condition characterized by severe and permanent hyperglycemia, comparable to type 1 diabetes state.

Insulin treatment

Insulin treatment began 24 h after streptozotocin injection in animals of group DBT+INS in a dose of 4-6 IU of Humulin NPH insulin (Eli Lilly y Compañía de México S.A., D.F., México) given as a

single daily subcutaneous (s.c.) injection each morning between 07.00 and 10.00 h. This treatment was adjusted to each animal's requirements every other day .

Tissue preparation and histomorphometric studies

The animals in all groups were euthanized on the 8th day. The upper maxillae were removed and fixed in buffered formalin (pH 7), decalcified in EDTA, and embedded in paraffin. Bucco-palatal sections at the level of the mesial root of the first upper molar were stained with H-E for histologic evaluation. Digital photographs using an objective of X40 magnification were taken and merged with software Photoshop 8.0 in order to reconstruct the vestibular plate of the alveolus. The area shown in Fig. 1 was used to carry out the following static histomorphometric studies (21,22) based on stereologic principles (23) using the Image Pro-Plus version 4.5 software (UTHSCSA). The parameters considered were:

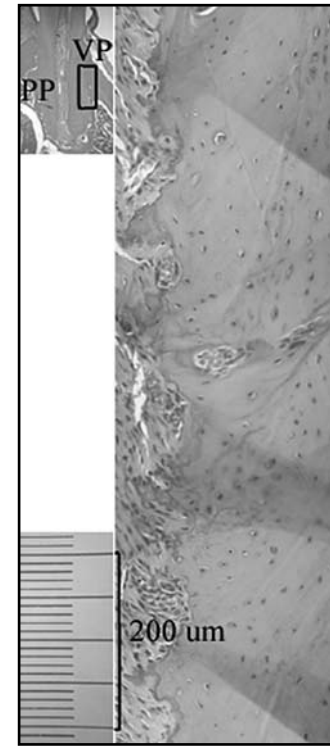

Fig. 1: *Digital microphotograph of a bucco-palatine section of the upper maxilla obtained at the level of the mesial root of the first molar. The middle third of the vestibular table (VP) was determined on the microphotograph and used to perform the histomorphometric evaluation of osteocytes, osteocyte lacunae and bone activity. H-E, 40X. VP: vestibular plate, PP: palatal plate.*

- ***Bone activity***:
  a) Erosive area (ES/BS) (%): fraction of total bone surface (BS) presenting erosive areas with or without osteoclasts
  b) Formation area (ObS/BS) (%): fraction of total bone surface presenting osteoid tissue and active cuboidal osteoblasts

- ***Total lacunar density*** (Tt.L.N/mm^2): total number of lacunae per mm^2. This parameter results from the sum of the following:
  - *Osteocyte density* (Ot.N/mm^2): number of osteocyte-occupied lacunae per mm^2
  - *Empty lacunar density* (EL.N/mm^2): number of empty lacunae per mm^2

- ***Lacunar Volume*** (Lac. Vol.): product of long and short axis diameters of all lacunae.

Lacunar shape: Lacunar shape was established in terms of the ratio between long and short axis diameters of each lacuna. Values between 1.0 and 1.5 were considered to correspond to round-shaped lacunae, values between 1.51 and 2.0 to intermediate lacunae and values above 2.1 to elliptic lacunae. A total bone area of approximately 24x10^3 µm^2 of the vestibular plate of the alveolus per animal was measured. Only lacunae that were completely included in the section thickness were measured (22). At least 80 lacunae were measured for each animal, based on the findings of other authors who established that the assessment of 50 lacunae or more guaranteed the validity of the technique (24).

Statistical analysis

Differences between variables were assessed by one-way ANOVA. Multiple comparisons were made using the Student-Newman-Keul's test. The level of statistical significance was set at $p<0.05$.

RESULTS

Effect of acute diabetes on bone activity

Bone surface activity values corresponding to the area shown in Fig. 1 revealed a significant increase in resorption areas in the DBT group against control ($p<0.05$). However, bone formation did not show significant differences between groups (Fig 2) .

Total lacunar density

Total lacunar density fell significantly in the experimental groups (DBT and DBT+INS) against control (C) and did not show significant differences amongst the experimental groups. This reduction in lacunar density occurred concomitantly with a sta-

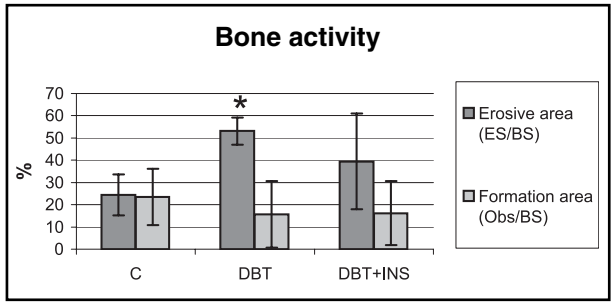

Fig. 2: *Bone activity in the vestibular plate (resorptive and formation areas) expressed as a percentage of the periodontal cortical in the area shown in Figure 1. The values are expressed as mean ± S.D. * means p<0.05.*

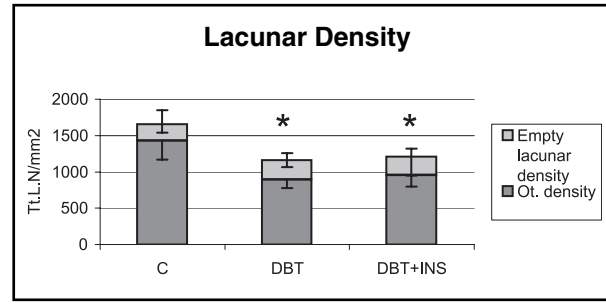

Fig. 3: *Lacunar density calculated as the sum of osteocyte density and empty lacunar density, expressed as number of lacunae/mm². Values are expressed as mean ± S.D. * means p<0.05.*

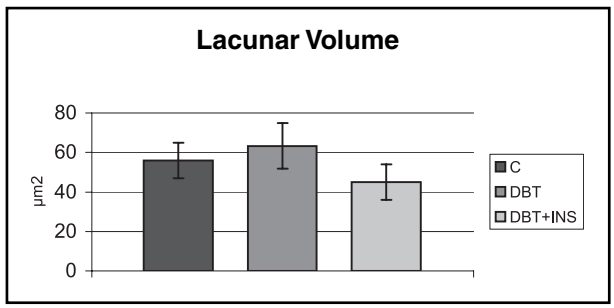

Fig. 4: *Lacunar volume expressed in µm². Values are expressed as mean ± S.D.*

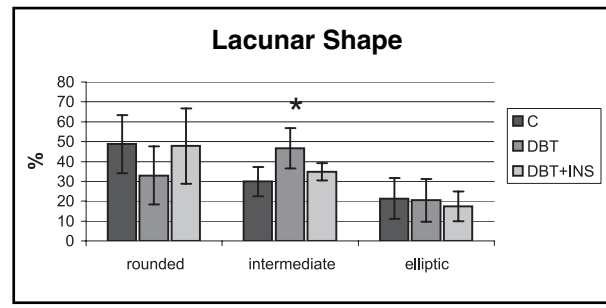

Fig. 5: *Lacunar shape determined by the relation between long and short axis diameters of each lacuna, expressed as a percentage. The values are expressed as mean ± S.D. * means p<0.05.*

tistically significant reduction in osteocyte density and an increase, albeit not statistically significant, in empty lacunar density, in the experimental groups (DBT and DBT+INS) against control (Fig 3).

Lacunar volume

Lacunar volume was slightly greater in the diabetic group (DBT) and slightly smaller in the treated diabetic group (DBT+INS) than in the control group (C). However, these differences did not reach statistical significance (Fig. 4).

Lacunar shape

Fig. 5 shows that the osteocytic lacunae in the diabetic group (DBT) tended to lose their round shape and adopt intermediate shapes, exhibiting a statistically significant increase against control ($p<0.05$) in the percentage of lacunae with intermediate shapes. Conversely, the lacunar shape in the DBT+INS group was similar to that in the control group.

DISCUSSION

The present study shows an early response of osteocytes to hyperglycemia. The experimental model of acute diabetes employed herein was used to study the early response of cells to acute hyperglycemia before systemic compensatory mechanisms are turned on. At this experimental time we observed an increase in resorption areas on the surface of the vestibular table (remodeling table) in the area under study in the DBT group.

The present results reveal a reduction in osteocyte density and an increase in empty lacunae. Normally, approximately 29% of the osteoblasts in cancellous bone differentiate to osteocytes, 6% to bone lining cells and the remaining 65% die by apoptosis (25). Diabetes causes alterations in osteoblast function, potentially resulting, in a reduction in the ratio of incorporation of osteoblasts to the matrix and interfering with the normal process of differentiation of osteoblasts to osteocytes. This process would result in a reduc-

tion in osteocyte lacunar density. Osteocyte density reflects the balance between the mean initial density when the bone is formed and the mean natural lifespan, reflecting the timing of death by apoptosis (26). The traditional interpretation of empty lacunae is that the remnants of cell death have been degraded and removed; an alternative interpretation is that the remnants persist for a long time but are easily dislodged during section preparation. In either case, an apparently empty lacuna is a reliable sign that the osteocyte that was originally present has died (26). Healthy bone exhibits some empty lacunae. The percentage of empty lacunae depends on the anatomic site (27) and on the passage of time but is independent of hormonal changes (28). A reduction in osteocyte lacunar density has been reported to occur with age in the cortical bone of human tibia (29). Microfractures associated to bone fragility rose in bones when lacunar density values fell below 600/mm^2 (29,30). It is important, therefore, when measuring lacunar parameters, to compare only those results obtained from homologous bones taken from subjects of the same age (3). Our data show that the normal value of lacunar density in the alveolar bone of young adult rats ranges between 1600 and 1800 lacunae/mm^2. These findings show that values may vary with bone site, subject and age. Interestingly, the present results show a reduction in lacunar density associated to an increase in the number of empty lacunae. This could only be due to the death of pre-existing osteocytes. The hormonal alterations induced by acute diabetes could have triggered processes of apoptosis of the osteocytes within the matrix that insulin administration was unable to revert and/or prevent. The fact that the lacunar volume did not vary could signify that hyperglycemia is not affecting the activity of the osteocyte *per se*. According to some authors (20), the shortening of cell processes observed by Transmission Electron Microscopy (TEM) would lead to alterations in the osteocytic network resulting in osteopenia. However, the identification of these alterations by TEM may be misleading. Confocal microscopy would contribute to the unequivocal determination of these alterations.

The alterations of the oseocytes and their lacunae described in the present study as an early response to hyperglycemia could contribute to the knowledge of the role of this cell in the process of adaptation involved in the development of osteopenia caused by diabetes. Future work devoted to unraveling these issues will involve long-term studies, the evaluation of apoptosis in osteocytes employing the TUNEL technique and dynamic histomorphometry studies to measure the rate of osteoblast formation.

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