Evaluation of apoptosis and osteopontin expression in osteocytes exposed to orthodontic forces of different magnitudes

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ABSTRACT

The in vivo response of osteocytes to different force magnitudes soon after they are applied remains to be elucidated. The aim of this study was to examine the early effects of applying a very light (LF: 0,16 N) and a very strong (SF: 2,26 N) orthodontic force during one hour on apoptosis and osteopontin (OPN) expression on alveolar bone osteocytes, in rats. Results: LF: compared to the control group, they showed a significant increase in OPN expression, and a significant decrease in the number of TUNEL-positive osteocytes. SF: compared to the control group, they showed a significant increase in OPN expression and a significant decrease in the number of TUNEL-positive osteocytes. Our results show that osteocytes respond very early to the application of tension and pressure forces of different magnitudes, and application of forces decreases the number of apoptotic osteocytes and increases OPN expression. These results allow concluding that osteocytes activate rapidly when subjected to locally applied forces, whether these forces be pressure or tension, light or strong forces.

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Key words: *osteocytes, mechanical stress, mechanotransduction, orthodontic tooth movement, apoptosis, osteopontin.*

Evaluación de la apoptosis y la expresión de osteopontina en los osteocitos luego de la aplicación de fuerzas ortodóncicas de diferentes magnitudes

RESUMEN

Hasta el momento no se ha dilucidado la respuesta temprana in vivo de los osteocitos a la aplicación de fuerzas de diferentes magnitudes sobre el hueso. El objetivo de este estudio fue examinar la respuesta temprana de la aplicación de una fuerza ortodóncica muy liviana (FL: 0,16 N) y muy fuerte (FF: 2,26 N) durante una hora sobre la expresión de apoptosis y osteopontina (OPN) en los osteocitos del hueso alveolar, en ratas. Resultados: FL: en comparación con el grupo control, mostraron un aumento significativo en la expresión de OPN y una disminución significativa en el número de osteocitos TUNEL-positivos. FF: en comparación con el grupo control, mostraron un aumento significativo en la expresión de OPN y una disminución significativa en el número de osteocitos TUNEL-positivos. Nuestros resultados muestran que los osteocitos responden muy temprano a la aplicación de fuerzas de tensión y presión de diferentes magnitudes, y la aplicación de fuerzas disminuye el número de osteocitos apoptóticos y aumenta la expresión de OPN. Estos resultados permiten concluir que los osteocitos se activan rápidamente cuando se los somete a fuerzas aplicadas localmente, ya sean estas fuerzas de presión o tensión, livianas o fuertes.

Palabras clave: *osteocito, fuerzas mecánicas, mecanotransducción, ortodoncia apoptosis, osteopontina.*

INTRODUCTION

Mechanosensation and transduction in osteocytes enables efficient exchange of physical and chemical signals among cells[1-3], mainly the signals that generate when bone is subjected to mechanical stress, as is the case of orthodontic forces applied to teeth[4].

Orthodontic tooth movement exerts different types of forces which have a different biomechanical effect: pressure and tension. It is assumed that the environmental changes in periodontal tissue during orthodontic tooth movement influence alveolar bone mechanically, acting on osteocyte activity and osteocyte network communication during the adaptive process[5-11]. Studies on alveolar osteocytes showed that application of tension forces during 48 hours was found to result in expression of connexin 43 protein[5], whereas application of pressure forces up to 24 hours resulted in a progressive increase in osteocytes undergoing apoptosis up to 1 day post-application, and a peak in the proportion of necrotic osteocytes and empty lacunae at 2 and 4 days respectively[6]. Results obtained using an experimental

model that combined both types of forces showed DMP-1 (dentin matrix protein-1) expression to increase after 6 hours and peak between days 3 and 7 post-application[7] and MEPE (matrix extracellular phosphoglycoprotein) expression to increase at 3 days[8]. According to reports in the literature, the percentage of osteopontin (OPN) positive osteocytes[9] and of connective tissue growth factor (CTGF) mRNA expressing osteocytes[10] also increased 12 hours after applying a force. Our previous studies showed that both the tensile and compressive forces exerted by orthodontia induced early changes in osteocytes and their lacunae, which manifested as an increase in lacunar volume and changes in lacunar shape and orientation, with an increase in canalicular width and increase in the length of cytoplasmic processes[11].

A study on *in vitro* response of osteoblastic cells to varying rates of fluid shear stress found that nitric oxide (NO) production was linearly dependent on the fluid shear stress rate, showing that strain rate (determined by the frequency and magnitude) is an important parameter for cell activation to stress[12]. However, *in vivo* osteocyte response to different magnitudes of orthodontic pressure and tension forces in terms of apoptosis and OPN expression has not been studied to date. The aim of this study was to examine the early effects of applying a very light (LF: 0,16 N) and a very strong (SF: 2,26 N) orthodontic force during one hour, on apoptosis and osteopontin (OPN) expression on alveolar bone osteocytes, in rats.

MATERIALS AND METHODS

Experimental Tooth Movement

Twenty four nine-week-old male Wistar rats, 220 g average body weight, were divided into groups of eight as follows: control group (C), a second group subjected to a 0,16N (16 gf) orthodontic force during 1 h (LF: light force) and a third group subjected to a 2,26 N (230 gf) orthodontic force during 1 h (SF: strong force). The appliance used to induce experimental tooth movement in the rats consisted of two stainless steel bands cemented to the upper first molars with a bracket welded to its palatal aspect through which a stainless steel wire spring was threaded[7,13]. Square section stainless steel wire (edgewise arch) was used to construct two springs designed to generate a 2,26 N (SF) and a 0,16 N (LF) respectively, towards the palatal aspect of the alveolus, exerting a compresssion force on the palatal site and a tensile force on the buccal site of the same alveolus (Fig. 1).

The animals were anesthetized by intraperitoneal injection of 50 mg/kg and 20 mg/kg body weight of 5% ketamine and 2% xylazine respectively prior to performing the procedures, and euthanized by ether overdose 1 h after applying the force.

All experiments were conducted in keeping with "The National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication 85-23, Rev 1985)". The experiment was approved by the Ethics Committee of the School of Dentistry, University of Buenos Aires (Res CD 015/14).

Tissue Preparation

Maxillae were resected and fixed in 4% formaldehyde in 0.2 M sodium phosphate buffer (PBS) at 4 ºC during 48 hours. The tissues were decalcified in 10% ethylenediaminetetraacetate (EDTA) pH 7.4 at 4 ºC for 60 days, and processed for embedding in paraffin. Five micrometer-thick bucco-palatine oriented sections were obtained at the level of the mesial root of the first upper molar. The specimens were oriented in the microtome under a stereoscopic microscope; they were sectioned to visualize the most axial plane of the mesial root in which the root canal and the apex are completely open, in a longitudinal orientation; the study sections were obtained at this level.

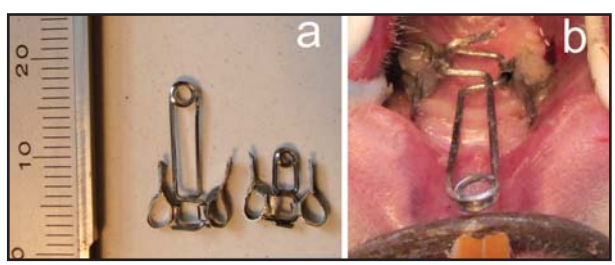

Fig. 1: Experimental orthodontic model. (a) Orthodontic springs: two different springs made of 0.016´´ X 0.016´´ stainless steel wire. Altering the diameter of the loop and the length of the spring allowed obtaining springs that exerted the force magnitudes used here, i.e. LF: 0,16N and SF: 2,26N. (b) Orthodontic appliance: two stainless steel bands were cemented to the upper first molars of experimental rats. Each band had a bracket welded to its palatal aspect through which a stainless steel wire spring was threaded. Because the springs exert force toward the palatal aspect of the alveolus, they exert a tension force on the periodontal aspect of the buccal wall, and a pressure force on the periodontal aspect of the palatal wall.

Detection of DNA fragmentation by TUNEL (transferase-mediated biotin-dUTP nick end-labeling)

The deparaffinized sections were treated with 20 µg/mL proteinase K (DakoCytomation, Carpinteria, CA, USA) in 10 mM Tris-HCL buffer, pH 7.4, at room temperature (RT) for 20 min, and then incubated with 0.30% H_2O_2 in methanol at RT for 30 min to block endogenous peroxidase activity. After rinsing with distilled water, the sections were incubated with TdT containing biotin-16-UTP buffer, pH7.2 (Chemicon, Temecula, CA, USA) at 37ºC for 90 min and then incubated with anti-digoxigenin antibody (Chemicon, Temecula, CA, USA), reacted with 3,3-diaminobenzidine (DAB) (Biogenex, San Ramon, CA, USA) and counterstained with methyl green. Sections of mammary gland after weaning were used as positive controls.

OPN Immunohistochemistry

The sections were incubated in 0.30% H_2O_2 in methanol for 30 min, followed by a wash in 10 mM phosphate-buffered saline (PBS) (pH7.2) during 10 min and incubated with 1% bovine serum albumin (BSA) (Sigma Chemical Co., St Louis, MO, USA) at RT for 30 min to block nonspecific binding of the antibody. The anti-OPN primary antibody (anti-rat OPN monoclonal antibody (Akm2A1:sc21742), Santa Cruz Biotechnology Inc., California, USA) was diluted 1:3000 in 10 mM PBS containing 0.10% BSA and 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA). The sections were incubated with the primary antibody at 4 ºC for 18 h. For control experiments, sections were incubated with non-immune rabbit IgG in place of the primary antibody. The primary antibody was detected using the avidin-biotin-peroxidase complex (ABC kit) (Biogenex, San Ramón, CA, USA) following instructions on the data sheet and reacted with DAB (Biogenex, San Ramón, CA, USA). The sections were counterstained with hematoxylin. Human bone marrow sections were used as positive control.

The number of TUNEL-positive osteocytes, the number of OPN-expressing osteocytes, and the proportion of OPN-expressing bone matrix were determined in an area measuring 100 µm wide and covering the full length of the periodontal aspect of both the buccal and the palatal walls of the alveolus. The immunolabeled sections were photographed using a 40X objective and imported into image analysis software for quantification. The number of TUNEL or OPN-positive osteocytes, defined as osteocyte cell bodies exhibiting brown staining, and the number of TUNEL or OPN-negative osteocytes, defined as osteocyte cell bodies exhibiting (methyl) green or hematoxylin staining respectively, were counted on each section. The percentage of immuno-labeled-positive osteocytes was calculated as the number of positive cells divided by the total number of osteocytes (positive and negative).

Statistical analysis

Results are shown as the mean ± standard deviation (SD). Data were compared using one-way analysis of variance (ANOVA) and Dunnett post-hoc test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Histological observations

At the experimental time points studied herein, the width of the periodontal ligament (PDL) was narrower on the palatine wall of the alveolus (pressure strain side) and wider on the buccal wall of the alveolus (tension strain side) in experimental groups compared to the control. This compression and stretching of the PDL was even along the full length of the corresponding wall and no hyalinized tissue was evident in any of the cases (Figure 2).

TUNEL detection of apoptosis.

A significant decrease in the number of TUNEL-positive osteocytes was observed on both the tension and pressure strain sides in the light-force (82.22% and 64.3% decrease respectively) and the strong-force (74.5% and 53.8% respectively) groups, compared to controls. The number of TUNEL-positive osteocytes was lower on the tension side in both experimental groups (Fig. 3).

OPN Immunohistochemistry

The number of OPN-expressing osteocytes was found to increase significantly on both the tension and pressure strain sides in both experimental groups compared with controls (148% and 172.7% increase corresponding to the light force and 117.6% and 116% increase in the case of the strong force). In addition, the proportion of OPN-expressing bone matrix was found to increase significantly on both

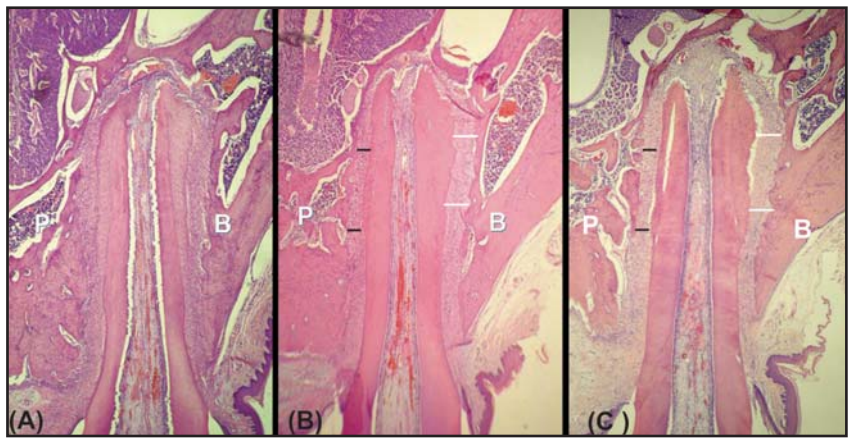

Fig. 2: Histological observations. Microphotographs of the mesial root of the first molar of a control rat (A) and experimental rats (B: LF; C: SF), showing the areas on the buccal side (tension strain side) and on the palatal side (pressure strain side) where we performed the immunohistochemical determinations. H-E,10X. White and black bars show stretching (B: buccal side) and compression (P: palatal side) of the PDL respectively occurred evenly along the full length of the corresponding wall. H-E, 10X.

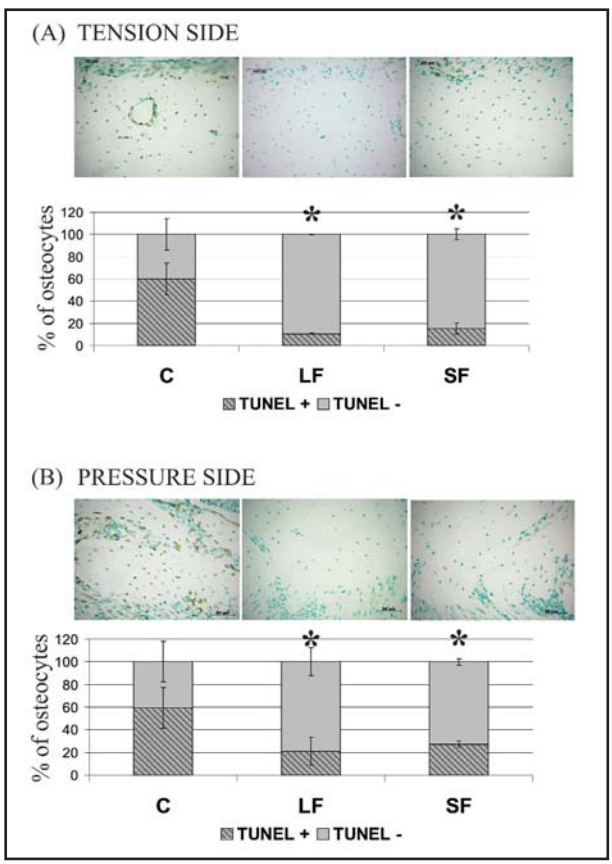

*Fig. 3: Immunohistochemical analysis of apoptosis using TUNEL. Tension (A) and pressure (B) strain sides. The figure shows that the percentage of TUNEL+ osteocytes decreased significantly on both the pressure and tension strain sides in animals under orthodontic forces compared to controls. Values are expressed as mean ± SD. *Statistically significant difference between control and experimental groups, $p<0.05$. SF: strong force, LF: light force, C: control. Representative microphotographs of paraffin-embedded sections immunostained for DNA fragmentation (brown) and counterstained with methyl green to show the number of osteocytes undergoing apoptosis.*

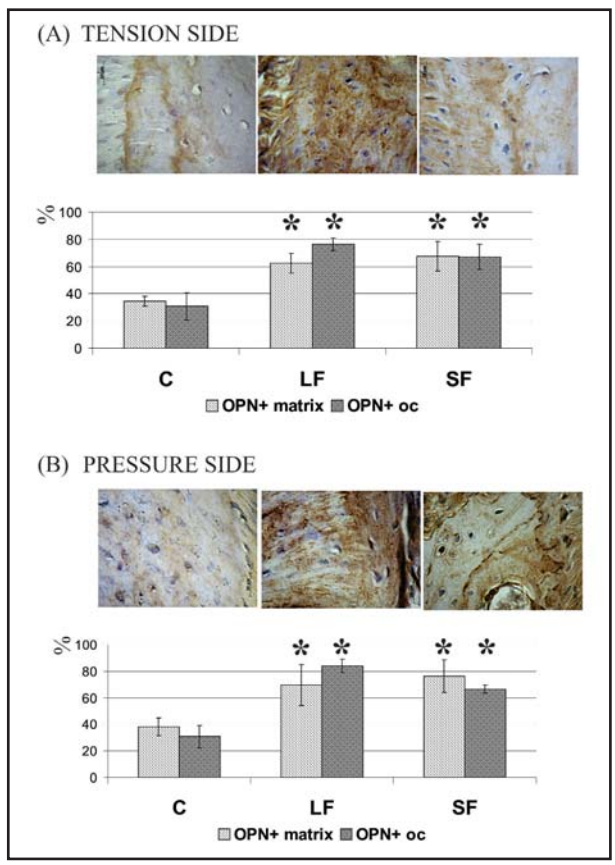

*Fig. 4: OPN immunostaining. Tension (A) and pressure (B) strain sides. The figure shows the percentage of OPN+ osteocytes and the percentage of OPN+ mineralized matrix. The percentage of OPN+ osteocytes increased significantly on both the pressure and tension sides in groups subjected to orthodontic forces compared to the control group; the increase was greater in those subjected to LF. The percentage of OPN+ mineralized matrix also increased significantly on both the pressure and tension sides in groups subjected to orthodontic forces compared to the control group. Values are expressed as mean ± SD. *Statistically significant differences between control and experimental groups, $p<0.05$. SF: strong force, LF: light force, C: control. Representative microphotographs of paraffin-embedded sections immunostained for OPN (brown) and counterstained with hematoxylin to show the overexpression of the protein both inside the cell and in the bone mineralized matrix.*

the tension and pressure strain sides in both experimental groups compared with controls (81.66% and 82.12% increase in the case of the light force and 96.85% and 100.16% increase in the strong force group) (Fig. 4).

DISCUSSION

This is the first study to evaluate the early *in vivo* response of alveolar osteocytes to different force magnitudes applied using an experimental model of orthodontic tooth movement. In this regard, the design of the spring used in this study allowed comparing the effect of a very light and a very strong force on both the tension and pressure strain sides. The orthodontic model used in the present study allows adapting the shape of the spring in order to vary the magnitude of the force and therefore analyze the biological effect of force magnitude on the cells involved in the adaptive remodeling process.

In the present study, osteocytes exhibited an early response to both tension and pressure forces of different magnitudes applied to bone. Specially, a significant decrease in the number of TUNEL-positive osteocytes was observed both in the light and strong force groups, with a marked diminution on tension strain sides. In other study[6], alveolar bone subjected to orthodontic forces exhibited a gradual increase in TUNEL positive osteocytes on the pressure side after 3h, peaking at 24 h. It is noteworthy however, that the authors encountered hyalinization in the periodontal ligament (PDL) on the pressure side where they performed the determinations, and they suggested that hyalinized PDL would impair transport systems resulting in ischemia or hypoxia, which would trigger osteocyte cell death. Conversely, though bearing in mind the differences between ours and their studies as regards the experimental conditions, our results showed neither an increase in the number of TUNEL-positive osteocytes nor hyalinization. It is therefore possible that since the mechanical stress in our experiment does not induce hyalinization of the PDL not impairing nutritional supply, osteocytes are prevented from entering apoptosis. In addition, it is known that forces exert an antiapoptotic effect. Shear stress reverses apoptosis of endothelial cells induced by different stimuli[14]. Moreover, shear stress upregulates the expression of integrins, and potential mechanotransducers located at the cell surface[15]. Various integrins prevent apoptosis[16,17] and it has been suggested that antiapoptotic integrin signaling involves the activation of the extracellular signal–regulated kinase (ERK) 1/2[18]. Several *in vitro* studies have shown that mechanical stimulation inhibits osteocyte apoptosis caused by serum-starvation[19], dexamethasone[20], and TNF-α[21]. Osteocytes detect fluid shear stress inside the lacuno-canalicular network via integrins[22-24], and NO production is initiated immediately[12]. NO prevents apoptosis of endothelial cells[25] and could be thought to have the same effect on osteocytes. NO production *in vitro* was found to be linearly dependent on fluid shear stress rate[12], and this may account for the differences, though not significant, in the percentage of the decrease in apoptosis observed between the force magnitudes used in the present study. Another response of osteocytes to mechanical loading is the immediate release of several growth factors[26]. By binding to cell-surface receptors, growth factors activate signaling pathways involving Bcl-2 family members that suppress apoptosis. In an unloaded model, apoptosis of osteocytes is associated with a transient decline in integrin and Bcl-2 survival protein levels[27]. Moreover, it has been demonstrated that mechanical stimuli preserve osteocyte viability via activation of ERKs and new gene transcription *in vitro*[20] and prevent glucocorticoid-induced apoptosis in MLO-Y4 osteocytic cells[28]. Even, a more recent study shows that mechanical stimulation for just 10 min is sufficient to trigger survival signaling in osteocytic cells[29]. In our work, mechanical reversion of osteocyte apoptosis was observed in osteocytes of groups subjected to orthodontic forces. It is therefore possible that NO production inside the lacuno-canalicular system, ERKs activation and autocrine effect of mechanical-induced growth factors secretion may be some of the mechanisms involved in protecting osteocytes from apoptosis, when subjected *in vivo* to mechanical stress exerted by orthodontic forces under the experimental conditions used herein. Based on the above, we conclude that forces within the physiological range seem to be an important survival factor for osteocytes at the experimental time points used in this study. Further *in vivo* studies must be conducted in order to confirm our observation that mechanical stimulation prevents, or at least delays osteocytes from entering apoptosis, triggering a

response that mediates activation of the remodeling process induced by the mechanical loads.

In our study, the potential association between force magnitude and OPN expression was evaluated on both the pressure and tension strain sides after applying orthodontic forces, showing a significant increase in OPN expression in all cases, and highest values in osteocytes subjected to the light force. OPN is considered to play an important role in bone remodeling since it is thought to promote or regulate the chemotaxis and attachment of osteoclasts to the bone surface during bone resorption[30]. Studies reported in the literature found that osteocytes located near resorption sites expressed OPN during physiological tooth movement[31], and almost all osteocytes expressed OPN 48 h after applying an orthodontic force, prior to osteoclast recruitment to the bone surface[9]. We found that OPN expression also increased significantly in the mineralized bone matrix of bone under stress, suggesting that this OPN was synthesized by pre-existing mature osteocytes and not by osteocytes recently included in the matrix, or by active osteoblasts located on the bone surface. This phenomenon suggests clear activation of osteocytes associated with communication via the lacuno-canalicular network inside the mineralized bone matrix. Interestingly, we also found that OPN expression also increased significantly in the tension side, where bone formation is stimulated. Accordingly with our results, Morinobu *et al* showed that OPN expression was enhanced during bone formation under tensile stress to calvarial sutures suggesting that the presence of OPN is one of the positive factors for osteoblastic bone formation in the suture under mechanical stress[32]. Our results demonstrate that tension and pressure forces of physiological magnitude applied *in vivo* seem to activate mechano-transduction and to promote the remodeling process via early expression of osteocyte OPN.

It is noteworthy that both pressure and tension forces were found to generate immediate osteocyte response. The marked decrease in apoptosis, and the significant increase in OPN expression observed soon after applying different forces (as regards type and magnitude) allow concluding that osteocytes activate very rapidly when subjected to locally applied forces *in vivo*, whether these forces be pressure or tension, light or strong forces, and lends support to the role of osteocytes as mediators of the activation of bone resorption and formation, which appear as the final response to the application of orthodontic forces.

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