Original Article

Expression of Nitric Oxide Synthases in Orthodontic Tooth Movement

Dorrin Nilforoushan[a]; Morris Frank Manolson[b]

ABSTRACT

Objective: To investigate differential expression of NOS isoforms in periodontal ligament (PDL) and bone in tension and pressure sides using a rat model of orthodontic tooth movement (OTM).

Materials and Methods: Immunohistochemistry with NOS isoform (iNOS, eNOS, and nNOS) antibodies was performed on horizontal sections of the first maxillary molars subjected to 3 and 24 hours of OTM. The PDL and adjacent osteocytes of the distopalatal root at pressure and tension areas were analyzed for expression of these proteins. The contralateral molar served as a control. Results were analyzed with one-way ANOVA and with two-way ANOVA.

Results: Expression of all isoforms was increased in the tension side. iNOS and nNOS expression in the pressure side with cell-free zone was decreased but in the pressure side without cell-free zone was increased. The number of eNOS-positive cells did not change, but the intensity of the staining was visibly increased in the tension side. Duration of OTM changed only the pattern of nNOS expression. Osteocyte NOS expression did not change significantly in response to OTM.

Conclusions: All NOS isoforms are involved in OTM with different expression patterns between tension and pressure sides, with nNOS being more involved in early OTM events. NOS expression did not change in osteocytes, suggesting that PDL cells rather than osteocytes are the mechanosensors in early OTM events with regard to NO signaling. (*Angle Orthod.* 2009;79:502–508.)

KEY WORDS: Nitric oxide synthase; Force; Osteocyte

INTRODUCTION

Nitric oxide (NO) is a short-lived free radical that is involved in regulating bone turnover and bone cell function[1,2]; it is a signaling molecule that mediates bone mechanical loading.[3] NO is generated by nitric oxide synthase (NOS) from oxygen and the amino acid L-arginine. Three isoforms of NOS have been identified: a neuronal form (nNOS),[4] an endothelial form (eNOS),[5] and an inducible form (iNOS).[6] Both nNOS and eNOS are expressed constitutively, and they are collectively referred to as constitutive NOS (cNOS).

An orthodontic force activates osteoclasts and osteoblasts, leading to bone remodeling and orthodontic tooth movement (OTM).[7] We and others have shown that NO is involved in OTM because tooth movement is decreased when NOS is inhibited and increased when NOS is stimulated.[8–10] In vitro, cyclic tension force activates NO production in human periodontal ligament (PDL) cells through eNOS,[11] while hydraulic pressure enhances NO production in cultured human PDL fibroblasts through nNOS.[12] In vivo, the expression of eNOS and iNOS in PDL decreases in an occlusal hypofunction model.[13] To date, the cell types that produce NO and the NOS isoforms responsible for OTM remain unknown.

We hypothesize that differential expression of NOS isoforms in the tension and pressure sides of the moved tooth may be responsible in part for bone resorption in the pressure side vs bone formation in the tension side. Toward this hypothesis, we set out to ascertain whether differential expression of the NOS isoforms occurs in PDL and bone in an animal model of OTM.

MATERIALS AND METHODS

Animal Model of OTM

Twenty-two male Spraque-Dawley rats (average weight, 250 ± 20 grams) received OTM for 3 (n = 10) and 24 (n = 12) hours of force. Under anesthesia, the

[a] Graduate PhD student, Orthodontic Department, Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada.

[b] Associate Professor, Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada.

Corresponding author: Dr Morris Frank Manolson, Faculty of Dentistry, University of Toronto, 124 Edward Street, Toronto, Ontario M5G 1G6 Canada
(e-mail: m.manolson@utoronto.ca)

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maxillary right molar was moved to the mesial by a 10 *grf* Sentalloy closed-coil spring (kindly provided by GAC International Inc, Bohemia, NY) running between right upper first molar and incisor. This spring delivers a constant force of 10 *grf.* The spring was fixed in place via ligature wires surrounding molar tooth and incisor. In the incisor area, because of lack of undercut, a cervical groove was prepared and the ligature wire was seated and secured by self-cure composite resin (kindly provided by Reliance Orthodontic Products Inc, Itasca, Ill) on both incisors.[8] This study was performed with a split-mouth design, with the contralateral side (left maxillary molar) of each animal serving as a control. Animals were acclimatized for 1 week prior to appliance placement and were fed a diet of soft laboratory food to minimize discomfort and risk of appliance displacement. The experimental design was approved by the Animal Ethics Committee of the University of Toronto.

Immunohistochemistry

Animals were euthanized after 3 or 24 hours of appliance insertion. Maxillae were dissected en bloc, fixed in 10% formalin overnight at 4°C, and placed in 4% EDTA at 4°C for 8 to 10 weeks of decalcification; the ethylenediaminetetraacetic acid (EDTA) solution was changed every other day. Fully decalcified samples (confirmed by radiographs) were dehydrated and paraffin embedded, and 6-micron-thick horizontal sections were prepared.

Sections obtained at the level of 500 to 600 microns from the furcation of the distopalatal roots of the maxillary first molars underwent immunohistochemical staining for NOS isoforms.[13] Briefly, the sections were dewaxed, endogenous hydrogen peroxidase blocked with 0.3% hydrogen peroxide in methanol for 30 minutes at 25°C, incubated with horse serum (R.T.U. Vectastain Universal Elite ABC kit, Vector Laboratories, Burlingame, Calif) for 1 hour at 25°C, then incubated with eNOS (1:50, Rabbit, BD Transduction Laboratories, Lexington, Ky, #610298), iNOS (1:100, Rabbit, Santa Cruz Biotechnology Inc, Santa Cruz, Calif, #sc-650), or nNOS antibody (1:100, Mouse monoclonal, BD Transduction Labs, #610308) overnight at 4°C. Negative controls were incubated with horse serum. Secondary horse antibody in 10% (v/v) rat sera (to prevent nonspecific staining) was added for 30 minutes, followed by Avidin-Biotin complex for 30 minutes (R.T.U. Vectastain Universal Elite ABC kit, Vector Labs). Diaminobenzidine (DAB, Vector Labs) was used to visualize the staining. Sections were counterstained with hematoxylin.

To identify osteoclasts, one slide per animal was stained for tartrate-resistant acid phosphatase (TRAP), as described previously.[14]

Area of Observation and Quantization

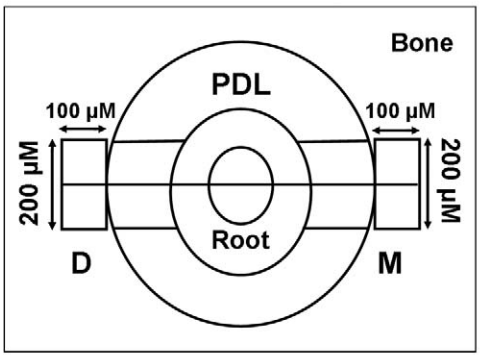
Images were taken with an Olympus BX51 microscope with an RT Color Spot digital camera (Diagnostic Instruments Inc, Sterling Heights, Mich). Mesial and distal areas of right and left maxillary molars were determined as described previously.[13] Three sections per isoform per animal were immunostained, and counting was performed on one section per isoform in each animal. One investigator performed all the counting, and reproducibility of the counting was 95%. This investigator was blinded to the experiment, but obvious visual differences between pressure and tension sides made it impossible for the investigator to be blinded to mesial and distal sides of OTM-treated teeth. Positive and negative cells were counted in four regions of interest (ROIs) on treatment and control sides. ROIs consisted of mesial PDL, mesial bone, distal PDL, and distal bone (shown in Figure 1A,B). The percentage of positive cells to total cells was calculated for each ROI. Figure 1C illustrates the total number of cells counted and the subsequent calculations performed from a representative experiment from one animal. To investigate NOS expression changes, percentage of positive cells to total of each ROI on the treatment side was compared with its control ROI with the use of paired *t*-tests (Statistical Package for the Social Sciences [SPSS], version 15.0, SPSS Inc, Chicago, Ill). To test whether changes in NOS expression from basal level were different in pressure and tension sides, percentage of positive cells was normalized to its control, and comparisons were made between tension and pressure sides through one-way analysis of variance (ANOVA) followed by the Tamhane and Games-Howell post hoc test (SPSS, version 15.0). To test whether duration of force (3 hours vs 24 hours) affected the NOS expression pattern in tension and pressure sides, two-way ANOVA with interaction (SPSS, version 15.0) was performed. Results were significant if $P < .05$.

RESULTS

Histologic Observations

Orthodontic force caused widening of the distal PDL and increased cell number. In contrast, mesial PDL was narrowed and cell number decreased (Figure 1B). Mesial PDL reacted in two different ways to orthodontic force. Following OTM, approximately half the animals had cell-free zones in the mesial PDL (indicated by arrows in Figure 2); in the other half, although cell numbers were decreased, cell-free zone did not ap-

A



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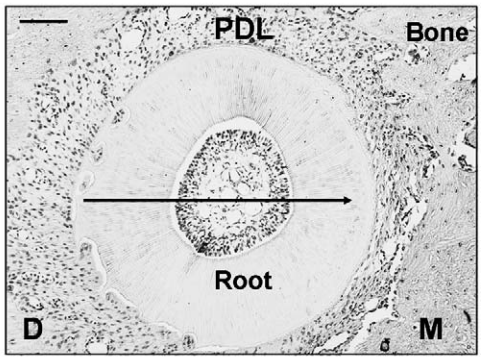

C

Area	PDL +ve cells	PDL total cells	% +ve /total PDL	+ve osteo-cytes	Total osteo-cytes	% +ve osteocytes /total
Control Distal	79	210	37.61	4	18	18.18
Forced Distal	152	337	45.10	6	18	37.5
Control Mesial	58	133	43.60	4	10	21.05
Forced Mesial	44	94	46.80	7	15	43.75

Figure 1. Regions of interest (ROIs) and cell numbers from a representative experiment. (A) ROIs are located at the mesial and distal periodontal ligament (PDL) and bone on the distopalatal root of the maxillary first molar. Bone ROI is a 200 × 100-µm² area, and PDL ROI is the PDL adjacent to the bone ROI. M indicates mesial; D, distal; and PDL, periodontal ligament. (B) Cross-section of distopalatal root of maxillary right molar, which received orthodontic tooth movement (OTM) for 24 hours and was stained with an inducible form of nitric oxide synthase (iNOS) antibody. Arrow shows the direction of tooth movement. The bar represents 100 microns. (C) To illustrate the average cell numbers counted per ROI and subsequent calculations, one representative sample stained with iNOS antibody is shown. +ve indicates positive.

pear. In the distal area of control and force sides, osteoclasts were observed at the junction of bone and PDL. Osteoclasts were not detected in the mesial area of control or forced teeth. Generally, osteocytes commonly expressed iNOS and nNOS, but rarely eNOS. All osteoclasts were positive for iNOS, nNOS, and eNOS expression. PDL cells commonly expressed iNOS and nNOS, but eNOS staining was mainly observed in PDL endothelial cells. Infiltration of acute inflammatory cells may contribute in part to increased cell number in the PDL tension side; therefore, a portion of the iNOS-positive PDL cells may be inflammatory cells.

iNOS Expression

iNOS expression increased in tension side PDL compared with its control at 3 and 24 hours of OTM (Figure 3A). As was mentioned earlier, animals were split between pressure sides with or without cell-free zone in the PDL. Different responses in NOS expression in these two subgroups made it essential for investigators to present the data separately. iNOS expression in the pressure side with cell-free zone was decreased, but in the pressure side without cell-free zone, it was increased at 3 and 24 hours. Although no difference was observed between tension and pressure sides without cell-free zone, an increase in the tension side compared with the pressure side was evident with cell-free zone at both times. Our two-way ANOVA with interaction analysis showed that the pattern of iNOS expression in tension and pressure sides did not change at between 3 and 24 hours.

With regard to osteocytes, iNOS expression did not change in the forced tooth compared with its control, and no difference was noted between pressure and tension sides at either 3 or 24 hours (Figure 4A).

nNOS Expression

nNOS expression increased in tension side PDL compared with its control at 3 and 24 hours of OTM (Figure 3B). nNOS expression in pressure side with cell-free zone was decreased, while in pressure side without cell-free zone, it was increased compared with its control at 3 and 24 hours. nNOS expression in ten-

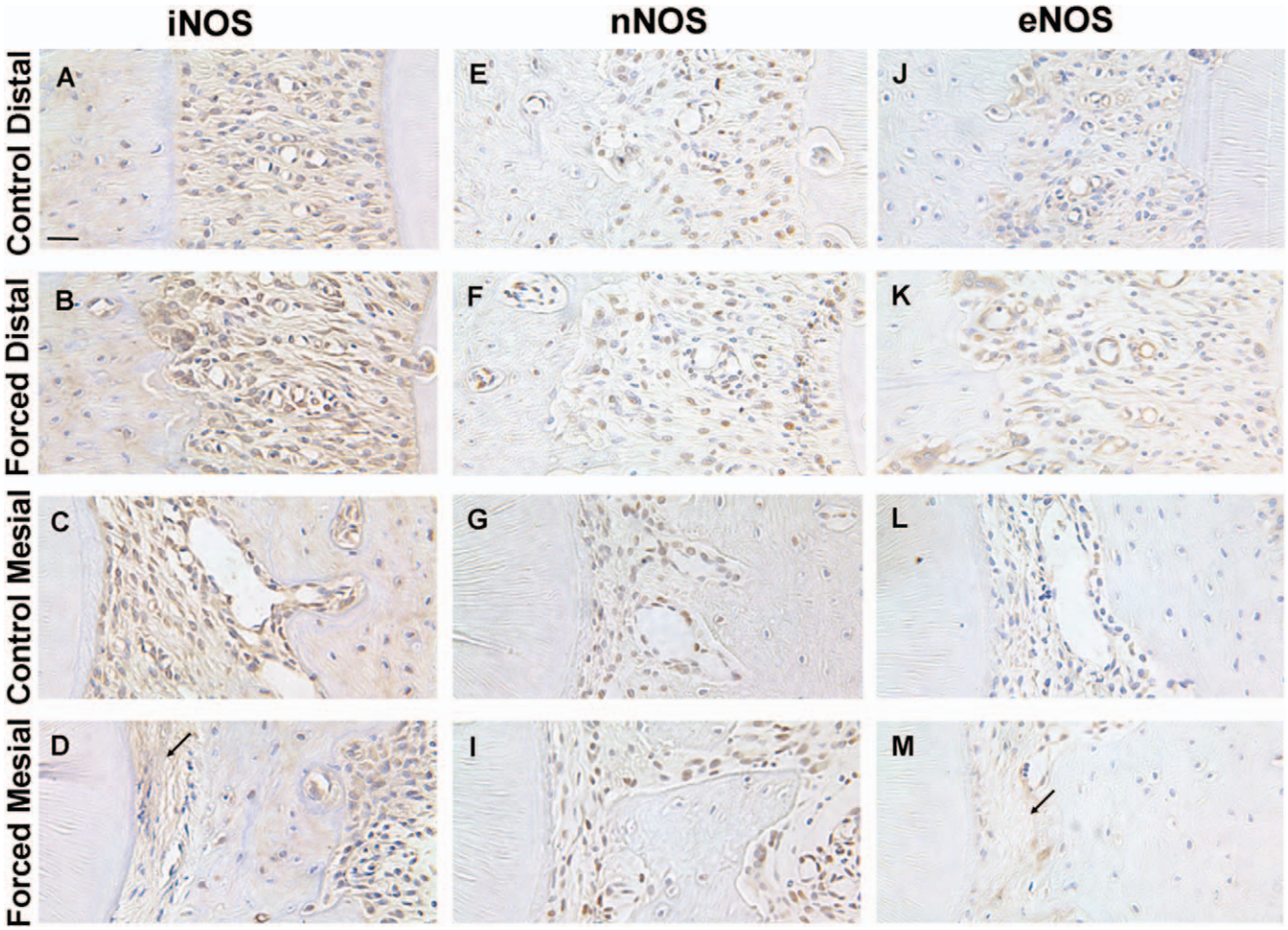

Figure 2. Inducible nitric oxide sythase (iNOS), neuronal nitric oxide synthase (nNOS), and endothelial nitric oxide synthase (eNOS) immunoreactivity in the periodontal ligament (PDL) and bone in distal (tension) and mesial (pressure) areas and their controls following 24 hours of orthodontic tooth movement (OTM). Six-micron sections obtained at the level of 500 to 600 microns from the furcation of the distopalatal roots of the maxillary first molars were incubated with iNOS (A-D), nNOS (E-I), and eNOS (J-M) antibodies and were visualized as described in Materials and Methods. Arrows point out cell-free zones. Bar in panel A represents 25 microns.

sion side was higher than in pressure side with cell-free zone at both 3 and 24 hours, while pressure side without cell-free zone was not statistically different from tension side. Two-way ANOVA with interaction analysis showed that the nNOS expression pattern changed over time; the difference in nNOS expression between tension and pressure sides with cell-free zone decreased significantly between the 3- and 24-hour time points.

With regard to nNOS expression in osteocytes, no significant difference was seen in the forced tooth compared with its control, nor between pressure and tension sides (Figure 4B).

eNOS Expression

As was mentioned earlier, eNOS expression in the PDL area was predominantly noted in endothelial cells and osteoclasts and was rarely observed in other PDL cell types. Following force application, the number of positive cells did not change, but the intensity of staining was visibly increased in tension side compared with control (Figure 2J through K). Because eNOS-positive osteocytes were rarely detected, quantization was impractical.

DISCUSSION

NO is one of the molecules that mediate mechanical signaling in bone. Osteocytes are known to be the mechanosensory cells in bone, producing NO in response to shear stress.[15,16] Osteoblasts also produce NO when subjected to mechanical strain and shear stress.[17] NO is involved in OTM,[8,9] but cell types and NO isoforms involved in OTM are not known. Here, for the first time, we show that with regard to NO signaling, PDL cells, rather than osteocytes, may play an important role in mediating force in early OTM events.

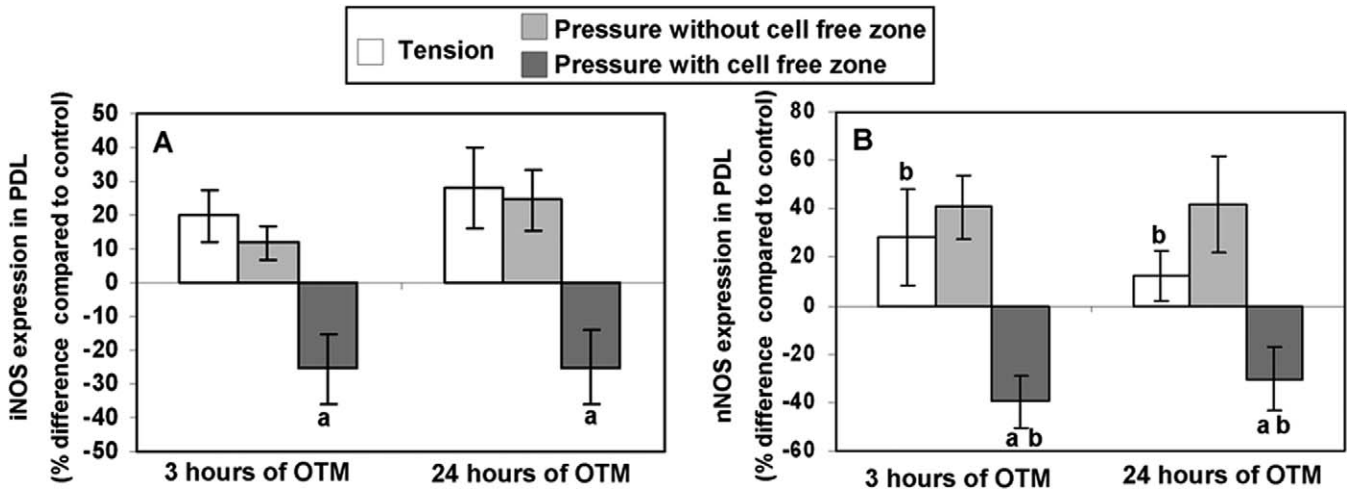

Figure 3. Inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) are differentially expressed in periodontal ligament (PDL) during orthodontic tooth movement (OTM). Percentage of positive (iNOS or nNOS) cells to total cell numbers was calculated in each region of interest (ROI), and the difference between the OTM side and the control side is presented as percentage difference compared with control. Tension and pressure (divided into two subgroups of pressure with cell-free zone and pressure without cell-free zone) data are presented for iNOS (A) and nNOS (B) at 3-hour and 24-hour time points. Data are presented as mean ± standard deviation (n = 10 for 3-hour time point and n = 12 for 24-hour time point). [a]$P < .05$ with one-way ANOVA of pressure with cell-free zone compared with its tension side. [b]$P < .05$ with two-way ANOVA with interaction of time and comparison of differences in nNOS expression between tension and pressure sides with cell-free zone at 3 and 24 hours.

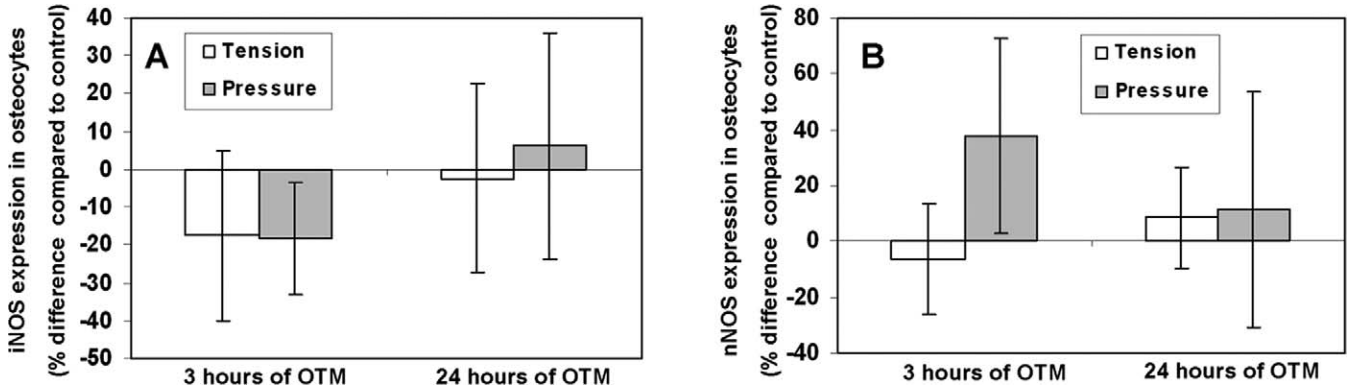

Figure 4. Inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) expression in osteocytes does not change between tension and pressure sides or over time during orthodontic tooth movement (OTM). Percentage of positive (iNOS or nNOS) osteocytes to total cell numbers was calculated in each region of interest (ROI), and the difference between OTM side and control is presented as percentage difference compared with control. Tension and pressure data are presented for iNOS (A) and nNOS (B) at 3-hour and 24-hour time points. Data are presented as mean ± standard deviation (n = 10 for 3-hour time point, and n = 12 for 24-hour time point).

To ensure that the results from our animal OTM model are clinically relevant, the maxillary molar was moved mesially with a constant force of 10 *grf*, shown to be comparable with the force used in human OTM.[18,19] To observe early events of NO involvement in OTM, 3- and 24-hour time points were selected. Although a dog model of OTM showed appearance of osteoclast in the mesial area after 24 hours of force application, in our rat model of OTM, we did not detect osteoclasts at the same time point in a comparable region.[20]

Our results show that expression of all three NOS isoforms was increased following force application in tension side. At both 3 and 24 hours, percentage of cells expressing iNOS and nNOS increased while eNOS staining visually intensified. This is consistent with a previous study on NOS expression in PDL, in which percentages of eNOS- and iNOS-positive cells were reduced following hypofunction and were returned to basal level after recovery.[13] Although no in vivo studies on nNOS expression in PDL have been conducted, consistent with our result in vitro, nNOS has been shown to be upregulated in human PDL fibroblasts in response to hydraulic pressure.[12]

Interpretation of results for NOS expression at mesial (pressure) side is complicated by reduced cell

number and appearance of cell-free zones. Cell-free zones have been reported to appear in response to light[21] and heavy forces.[7] Appearance of cell-free zones in about half of our animals illustrates variability of the cellular response to constant force and duration. We observed that iNOS and nNOS expression increased in animals without cell-free zones and decreased when cell-free zones appeared. It is interesting to note that most of the cells remaining in the ROIs containing cell-free zones were negative for iNOS and nNOS. Because high NO concentration leads to cell death,[22] we hypothesize that prior to 3 hours in these animals, an increase in NO concentration and subsequent cellular apoptosis resulted in cell-free zones. Remaining cells in these ROIs would be negative, thus producing the observed decrease in iNOS and nNOS expression. NOS expression at earlier time points could confirm this hypothesis. Another approach may include use of the NOS inhibitor in an OTM animal model and investigation of the prevalence of cell death and cell-free zones.

With respect to expression differences over time, nNOS was found to exhibit a greater difference between tension and pressure at 3 compared with 24 hours, suggesting that nNOS may be more deeply involved in early events of OTM. In contrast, we found no differences in iNOS expression between the two time points.

We could detect no significant changes in NOS expression in osteocytes visually or statistically. Furthermore, positive osteocytes for eNOS were rarely detected. In contrast, iNOS and eNOS have been shown to be upregulated in osteocytes in a hind limb rat model with 2 weeks unloading followed by 1 week reloading.[23] This may suggest that 24 hours is insufficient time for a response from osteocytes. Alternatively, osteocytes may not be involved in sensing the orthodontic force applied to the tooth. In support of this, it has been shown that in response to force, while eNOS expression in osteocytes residing in long bones increase,[24] a similar response is not seen in calvaria.[25] One of the main differences between OTM and forces applied to long bones is the presence of the PDL complex, which modifies transfer of force to the bone.

CONCLUSIONS

- PDL cells respond to OTM by producing NO. All three NOS isoforms are involved in OTM, with different expression patterns seen between tension and pressure sides, and with nNOS being more involved in early OTM events.
- NOS expression did not change in osteocytes, suggesting that PDL cells, rather than osteocytes, are the mechanosensors in early OTM events, with regard to NO signaling.

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