Original Article

The Role of Nitric Oxide in Orthodontic Tooth Movement in Rats

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Abstract: Nitric oxide (NO) is involved in second messenger formation, osteoblast and osteoclast function, and pulpal blood flow. This raises the question of whether or not altered NO production interferes with orthodontic tooth movement (OTM) by influencing the bone remodeling cycle. To investigate the role of NO in OTM, a rat model was established and 48 rats were divided into four study groups of 12 rats each. A 5 mm nickel-titanium closed-coil spring was ligated between the right maxillary incisor and first molar of each rat to deliver an initial force of 60 g. A saline group received subperiosteal injections of normal saline (50 μL/kg), an L-arginine (L-arg) group received L-arginine (NO precursor) injections (200 mg/kg), and a, L-NAME group received N^G-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor)(10 mg/kg) injections. All injections were given in the upper right first molar mucosa from the first through the 11th day of force application at 48-hour intervals. A control group received no injections. Tooth movement measurements were done at the time of injections. Animals were sacrificed 13 days after appliance insertion and final OTMs were measured at the time of sacrifice. From the third day till the end of the experiment, the L-arg group showed a significant increase in tooth movements, whereas the L-NAME group showed a significant decrease in tooth movements compared to the control and saline group ($P < .001$). Histopathologic studies revealed that the number of osteoclasts was significantly higher in the L-arg group smears, while the number of osteoclasts in the L-NAME group was significantly lower as compared to the control group ($P < .001$). Scanning electron microscope analysis showed that the force-induced root resorption in the L-arg group was less than the control group. This study suggests a role for NO in the bone remodeling cycle. (*Angle Orthod* 2002;72:211–215.)

Key Words: Orthodontic tooth movement; Nitric oxide; L-arginine; L-NAME; Rat

INTRODUCTION

Reducing orthodontic treatment time is a primary goal for orthodontists.[1] Since orthodontic tooth movement (OTM) is performed via bone resorption and apposition, factors influencing these processes may enhance OTM.[1] For instance, we have recently shown enhancement of OTM in animal models of hyperthyroidism and secondary hyperparathyroidism.[2,3] Orthodontic forces, bioelectric changes, local hormones, and a variety of drugs act as the first messenger to promote production of intracellular second messengers such as Ca^{2+}, cGMP, or cAMP.[4] Thus, it is logical to suggest that alterations in second messenger formation can affect the rate of OTM.

In 1978, it was found that nitric oxide (NO) is produced from L-arginine amino acid by nitric oxide synthase (NOS), which is present in endothelial cells and other tissues.[5] Studies revealed the role of NO in relaxation of smooth muscles of arteriole walls, inhibition of platelet aggregation, and interneuronal transaction.[5,6] Nitric oxide is released from macrophages and osteoclasts during cell to cell interactions and acts as a cytotoxic molecule to kill intracellular microorganisms and tumor cells. Nitric oxide also plays a role in bone resorption.[7]

It is suggested that NO mediates the pulpal inflammatory response through its effects on the paralesional pulpal tissue and surrounding endothelial and other vascular structures.[8] It has recently been found that cultured human periodontal ligament fibroblasts enhance their production of basal nitric oxide by the application of hydraulic pressure.[9]

Because NO elevates the amount of cGMP, the second

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messenger in the bone remodeling cycle, it is suggested that NO may raise the rate of OTM leading to altered orthodontic treatment duration. The aim of this study was to observe the role of NO in OTM using NO precursor (L-arginine) and NOS inhibitor L-NAME (N^G-nitro-L-arginine methyl ester).

MATERIALS AND METHODS

Animals

Forty-nine male Sprague-Dawley rats with an average weight of 250 ± 20 g obtained from the Razi Institute, were used in this study. Animals were acclimatized for one week in plastic cages with a standard 12-hour light-dark cycle. The animals were fed a diet of soft laboratory food to minimize any discomfort to the animal after orthodontic appliance insertion and to minimize the risk of appliance displacement. The study conformed to the *Guide for the Care and Use of Laboratory Animals* published by US National Institutes of Health (NIH Publication No 85–23, revised 1985). This study was performed as a split-mouth design with the contralateral side of each animal serving as its control. The rats were randomly divided into four study groups (N = 12 in each group). The groups included: (1) a control group where no injections were performed; (2) a saline group that received normal saline (50 μL/kg) injections; (3) an L-arg group in which injections of L-arg (Fluka, Buchs, Switzerland) (200 mg/kg) were performed; and (4) an L-NAME group receiving L-NAME (Sigma, St. Louis, MO)(10 mg/kg) injections. All injections were administered as local subperiosteal injections in the buccal mucosa of the upper right first molar at 48-hour intervals starting from the first day of appliance insertion to the 13th day (end of the study). In order to make the injections possible, the animals were made drowsy by a brief administration of ether before each injection. In addition to the study groups, one additional rat was considered as normal. The normal rat received neither injection nor appliance insertion during the study. Later it was used in the scanning electron microscope analysis.

Orthodontic treatment and OTM measurements

Each rat was anesthetized with an intraperitoneal injection of Ketamine 50 mg/kg body weight (Ketamine Hydrochloride, Gedeon Richter Ltd, Budapest, Hungary) and Xylazine Hcl 6 mg/kg body weight (Rompoun, Bayer, Leverkusen, Germany). The orthodontic appliance was a replication of the appliance presented by King and Fischischweiger.[10] Orthodontic force was applied by a 5 mm length of 0.006 × 0.022″ Nitinol closed-coil spring (3M/Unitek Hi-T II, Monrovia, Calif) running between the right upper first molar and incisor. The spring was fixed in place via 0.010″ steel ligature wires (Dentaurum, Newtown, Penn) surrounding the molar tooth and incisor. Due to lack of undercuts in the incisor area, a cervical groove was prepared on the tooth in which the ligature wire was seated and secured with a composite resin (Self cure Degufill, Degussa AG, Frankfurt, Germany) on both incisors. Each spring was activated once according to Heller and Nanda's[11] method to produce 60 g of force. The force was applied for the 13 days during which the injections were done. Orthodontic tooth movements (OTM) were measured at the time of each injection (days 1,3,5,7,9,11), using a Filler Gauge (Mitutoyo Co, Kawasaki-Shi, Japan) directly in the mouth to reveal the distance between the first and second right molars. This distance was initially zero in the samples. Thirteen days after appliance insertion, animals were sacrificed using an ether overdose. An additional silicone impression (President; Liechtenstein) was taken and poured with ultra strength dental stone (Gildand, Germany) before removing the appliance to prevent any potential relapse of tooth movement. The final measurement was performed using a Filler Gauge on the plaster replica. The same operator performed all measurements.

Scanning electron microscope examination

To investigate the resorptive changes on the molar root surface, scanning electron microscope examination was performed on the mesial surface of the mesial root of the maxillary right first molar. The molar of the normal rat was used to serve as a normal root surface. One rat from each study group was randomly selected. Each specimen (maxilla and corresponding teeth) was placed in a 4% sodium hypochlorite solution for one week. Then the right upper first molar was extracted and left to air dry for one day and placed on the examination stub. Sites to be examined were coated with a layer of gold. Resorption patterns in different study groups were observed and compared by scanning electron microscope.

Histological evaluation

To observe histological changes of bone and tissue surrounding the tooth, the posterior right hemimaxilla containing three molar teeth, bone, and soft tissue was dissected and immersed in 10% formalin solution for 10 days. The samples were washed with water and placed in 5% formic acid for one week in order to make the bone soft enough to cut. Samples were seated in paraffin and five-micrometer thick mesiodistal sections were cut and every fifth section was stained with Hematoxylin and Eosin. The osteoclast count, inflammation and bone resorption were the criteria for comparison with the samples.

Statistical analysis

Data are shown as mean ± standard error of the mean (SEM). Statistical evaluation of the data was done with the analysis of variance (ANOVA) followed by the Newman-

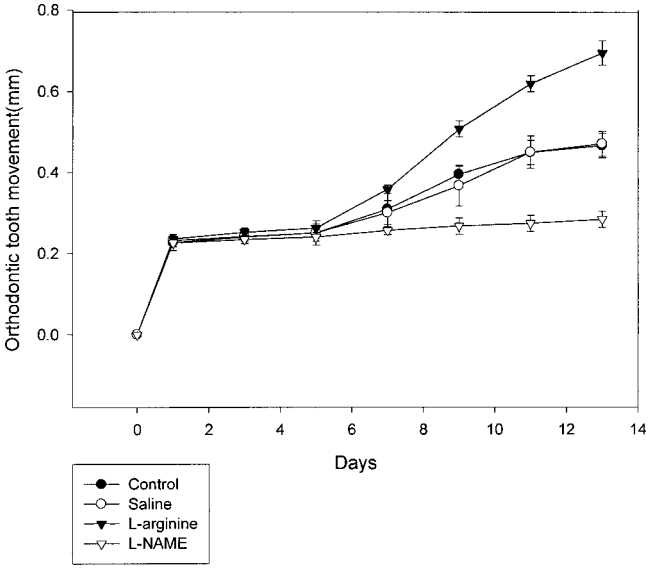

FIGURE 1. The orthodontic tooth movement-time curves for four study groups. *$P < .001$ for measurements from the third day until the end of the experiment in L-arg and L-NAME groups compared to control and saline groups. Data are shown as mean ± standard error of mean. Sample size was 12 in each study group.

Keuls test for multiple comparisons and a *P*-value less than .05 was considered statistically significant.

RESULTS

OTM results

All appliance-treated molars showed evidence of tooth movement. No tooth movement was noted in the no appliance side. OTM-time curves for study groups are shown in Figure 1.

Scanning electron microscope results

Resorptive lacunae were observed on the mesial surface of all first molar mesial roots, even in the normal rat (the rat with no force application or injection). Root resorption in the control and saline groups was greater than in the normal rat. Root resorption in the L-arg group was less than in the control group. Root resorption in L-NAME group was near the level observed in the control group.

Histological results

No abnormalities were observed in either the control group or the saline group. Periodontal ligaments in the study group appeared normal. In the normal rat, osteoclasts were rarely observed. On the other hand, osteoclasts, inflammatory cells, and recently formed bone and resorptive areas were seen in the study groups.

Compared to the control and saline groups, more inflammatory cells, osteoclasts, and bone resorption were seen in the L-arg group and less in the L-NAME group. Table 1 shows the osteoclast counts of the four groups.

TABLE 1. Number of Osteoclasts in a Field of 400 × 800 μm² for Control, Saline, L-arg, and L-NAME Groups

Study Group[a]	Number of Osteoclasts in a Field of 400 × 800 μm²
Control	1.84 ± 0.12
Saline	8.50 ± 0.65
L-arg	16.32 ± 1.03*
L-NAME	6.38 ± 0.75*

[a] N = 12 in each study group (one rat in each study group was used in scanning electron microscope analysis). Data shown as mean ± standard error of mean (SEM).
* $P < .001$ compared with control group.

DISCUSSION

Compared to the control group, OTM in the L-arg group was increased, but it was significantly decreased in the L-NAME group ($P < .001$). These differences are significant from the third day until the end of the study period. On the other hand, no significant difference was observed between the control and saline groups during the study ($P < .001$).

The control group curve is identical with the curve of OTM as presented in previous studies. It consists of three different parts. The first phase involves rapid tooth movement in the periodontal ligament area at the end of the first day. Tissue compression by an orthodontic force causes rapid movement after force application. The second part of the curve is a slow movement, taking place from day 2 to day 5. During this period, osteoblasts and osteoclasts cause remodeling of the bone. From day 6 until the end of force application the movement is rapid and again reveals a high turnover in bone.[12]

The number of osteoclasts in the L-arg group was significantly higher than that in the Saline and L-NAME groups ($P < .001$). This is in accordance with our OTM findings. Since the same orthodontic appliance applied the same 60 g mesial orthodontic force in all four groups, the differences among groups are attributed to the injections (NO precursor and NOS inhibitor), which led to enhancing or inhibiting NO production. Therefore, we suggest that NO production may have led to faster OTM in our samples corroborating previous reports in the literature.[13]

Studies show that various factors might influence the rate of OTM. In one study an increased vascular perfusion and macrophage count were found during OTM in mice.[14] Another study on the rat's periodontal vascular system revealed that vessels surround hyalinized areas during OTM and that blood flow rises in the areas with osteoclastic activity with macrophages and monocytes present in large numbers during bone resorption.[15]

Davidovitch measured cGMP and cAMP levels in the alveolar bone of cats under orthodontic treatment and re-

ported that Ca^{2+}, cGMP and cAMP act as key mediators or second messengers in the function of many drugs and hormones.[16] Igarashi et al[17] and King et al[18] reported that intracellular second messenger plays an important role in the differentiation of osteoclasts from monocytes in bone resorption during mechanical orthodontic force application.

Nakago-Matsuo et al[9] applied hydraulic pressure on cultured human periodontal ligament fibroblasts and reported that fibroblasts produced a significantly larger amount of NO pressures of 75 and 100 mm Hg. The pressure level applied to enhance NO production was comparable to the magnitude of clinically used orthodontic forces (60–80 g/cm^2).[19,20]

It has been suggested that orthodontic forces may elevate NO production from periodontal ligament fibroblasts, which then activates guanylyl cyclase in periodontal ligament fibroblasts, leading to an increased level of cGMP.[6,21] This second messenger in cell cytoplasm raises lysosome membrane permeability, leading to exocytosis of lysosome content resulting in resorption of organic and mineral elements of bone. Furthermore, NO synthesizes prostaglandins by direct activation of cyclooxygenase.[22] Nitric oxide may also diffuse into the dental pulp and reach the endothelial cells of vessels, changing the vascular tone. [23] Nitric oxide may diffuse to the alveolar bone and influence the function of osteoclastic differentiation.[24] Furthermore, osteoblast function is modulated by a NO-dependent mechanism.[25] The effect of NO on the osteoblast is mediated by the second messenger cGMP.[26] Thus, NO plays a role in bone resorption as well as bone formation and facilitates these phenomena during force application. This may lead to decreased root resorption of the teeth undergoing orthodontic treatment.

CONCLUSIONS

Injections of L-arg (nitric oxide precursor) and L-NAME (nitric oxide synthase inhibitor) during orthodontic tooth movement in rats resulted in:

1. Increases in NO production, which increased bone remodeling, and OTM, while a decreased NO production led to a decreases in OTM.
2. NO decreased root surface resorption.
3. NO increased the number of osteoclasts surrounding the tooth undergoing orthodontic tooth movement and inhibition of NO production had an opposite effect on the number of osteoclasts.

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