EFFECT OF NITRIC OXIDE (NO) ON ORTHODONTIC TOOTH MOVEMENT IN RATS

By

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2 MATERIALS AND METHODS</td>
<td>7</td>
</tr>
<tr>
<td>Animals</td>
<td>7</td>
</tr>
<tr>
<td>Radiographic Examination</td>
<td>8</td>
</tr>
<tr>
<td>Histological Examination</td>
<td>9</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>9</td>
</tr>
<tr>
<td>3 RESULTS</td>
<td>11</td>
</tr>
<tr>
<td>4 DISCUSSION</td>
<td>15</td>
</tr>
<tr>
<td>LIST OF REFERENCES</td>
<td>18</td>
</tr>
<tr>
<td>BIOGRAPHICAL SKETCH</td>
<td>20</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Synthesis and metabolism of NO</td>
<td>6</td>
</tr>
<tr>
<td>2.</td>
<td>Change in body weight (gm) of rats receiving L-NAME at three distinct time points: surgery, activation and sacrifice</td>
<td>13</td>
</tr>
<tr>
<td>3.</td>
<td>Change in body weight (gm) of rats not receiving L-NAME at three distinct time points: surgery, activation and sacrifice</td>
<td>13</td>
</tr>
<tr>
<td>4.</td>
<td>Consumption of L-NAME (ml) by the control and springs groups</td>
<td>13</td>
</tr>
<tr>
<td>5.</td>
<td>Orthodontic tooth movement from Day 1-10 of spring rats vs. spring rats receiving the NOS inhibitor</td>
<td>13</td>
</tr>
<tr>
<td>6.</td>
<td>Orthodontic tooth movement from Day 1-10 of molar drift rats vs. molar drift rats receiving the NOS inhibitor</td>
<td>14</td>
</tr>
<tr>
<td>7.</td>
<td>Photomicrographs showing the distal root of the maxillary first molar</td>
<td>14</td>
</tr>
</tbody>
</table>
Abstract of Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Master of Science

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By

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Applying force to a tooth produces strains in the periodontal ligament (PDL) and the surrounding bone. The strain initiates an acute inflammatory response that induces the secretion of prostaglandins, cytokines, and growth factors. These factors in turn produce an uncoupling in the normal bone remodeling process, which then produces tooth movement. Nitric oxide (NO), a short-lived free radical, is produced by enzymes called NO synthases (NOS). Nitric oxide has important effects in bone remodeling. The purpose of this study was to examine the effect of nitric oxide on orthodontic tooth movement in rats. Maxillary first molars of male Sprague-Dawley rats were moved via a closed-coil spring for 10 days. Systemic administration of N-nitro-L-arginine methyl ester (L-NAME), a general inhibitor of nitric oxide synthases, significantly reduced tooth movement. These results suggest that NO levels may influence orthodontic tooth movement.
CHAPTER 1
INTRODUCTION

Among the many goals of the specialty of orthodontics is for the clinician to achieve tooth movement in an effective and expedient manner. It is quite difficult to move a tooth spatially without having an impact on neighboring teeth; and so the most essential element to the practice of orthodontics is anchorage. Absolute anchorage would imply that one tooth is moved without any untoward effects on the adjacent teeth being used for that particular movement. Until now, clinicians have relied heavily on laboratory-constructed devices such as the transpalatal arch (TPA) or Nance to maintain anchorage within the dental arch. Additionally, clinicians have sought the help of biologically invasive dental implants. With both approaches, the results have been less than satisfactory. In the quest for more complete anchorage, research has focused on the field of pharmacology. Recently, bisphosphonates have been used as a way to prevent tooth movement (Igarashi, Mitani, Adachi, Sinoda 1994). Histologic examination has shown that with the application of bisphosphonates in experimental animals, fewer osteoclasts appeared on the alveolar bone surface and that bone resorption was inhibited (Igarashi et al.1994). Thus, a pharmacological approach seems promising in the endeavor to gain pure control of tooth movement.

Orthodontic tooth movement occurs as a response to mechanical forces placed on the tooth that disperse throughout the periodontal ligament and then initiate bone remodeling. This sequence of events has previously been described by the pressure:tension hypothesis (that bone is resorbed in areas of pressure and is formed in
areas of tension) (Sandy, Farndale, Meikle 1993). Applying force to a tooth disrupts the equilibrium between bone formation and resorption. This results in an overwhelmingly greater amount of bone resorption than formation on the pressure side; and the reverse on the tension side (Sandy et al.1993).

Orthodontic tooth movement actually involves three unique phases (King, Keeling, Wronski 1991). As an orthodontic force is applied to a tooth, the initial phase allows for some movement of the tooth. However, this movement is strictly compressive deformation of the periodontal ligament on the pressure side. In the second phase, the lag phase, there is no tooth movement. Here, necrotic tissue that is accumulated in the compressed area of the PDL is eliminated (King, Keeling, McCoy, Ward, 1991). Essentially, hyalinization occurs in almost all cases. Elimination of the hyalinized tissue is associated with undermining bone resorption (King et al., 1991). The final phase is when true orthodontic tooth movement occurs. Osteoclasts within the PDL spaces begin to resorb the adjacent alveolar bone and permit the movement of teeth (King et al., 1991).

Mechanical stress is believed to influence both bone formation and resorption. The immediate effect of force on a tooth is movement of the tooth within the PDL space that stimulates nerves and causes blood-flow changes (Ehrlich, Lanyon 2002). Any of these events could transform the physical force into a message that activates the biological system. Damaged tissue releases chemo-attractants that elicit the response of macrophages and PMNs (to remove the necrotic debris). These cells release factors (called chemokines and cytokines) that allow them to communicate with one another. These factors may cause pleuripotent stem cells to differentiate and thus augment the bone remodeling process (Norton 2000).
Bone remodeling occurs through the activation of specific signaling pathways. Inflammatory cytokines produced by mechanically activated cells; and a variety of local mediators have been implicated in modulating the activities of osteoclasts and osteoblasts. However, the signal-transduction pathways of orthodontic mechanical stimuli have yet to be elucidated (Hayashi, Igarashi, Miyoshi, Shinoda, Mitani 2002). It is quite possible that the mechanical signal itself may be stimulatory. Osteoblasts and osteocytes may themselves act as mechanical sensors for bone remodeling (Hayashi et al. 2002). It has been previously shown that osteoblasts lay down new bone matrix in the form of osteoid in areas formerly occupied by osteoclasts (King et al. 1991). Frequently, osteoblasts become trapped within the bone matrix; and differentiate into osteocytes. These osteocytes communicate with each other and with cells on the bone surface through cytoplasmic processes throughout the bone matrix. It is thought that osteocytes act as sensors of mechanical stress by detecting and responding to changes in fluid flow (Van’T Hof, Ralston 2001). From in vitro and in vivo studies, the orthodontic force (mechanical stress) appears to evoke an acute inflammatory response mediated by the release of prostaglandins, cytokines and growth factors (Sandy et al. 1993). These factors, in turn, regulate bone remodeling.

Nitric Oxide (NO) is a short-lived, reactive molecule that serves as a sensitive mediator in the nervous, vascular and immune systems. It is produced by nitric oxide synthase (NOS) from the L-arginine amino acid and yields L-citrulline as a co-product (Figure 1) (Chae et al. 1997). Many cell types within the bone microenvironment are potentially capable of producing NO (Evans, Ralston 1996). Nitric oxide synthesis can
be induced in osteoblasts and osteocytes when exposed to mechanical strain and/or shear stress (Van’T Hof et al. 2001).

Three forms of NOS are currently recognized: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) (Evans et al. 1996). The names reflect the tissues in which these enzymes were first located yet they may be found in a variety of tissues and cell types. The neuronal and endothelial forms are constitutively expressed and their activity depends on elevated levels of intracellular calcium (Ralston et al. 1995). The inducible pathway of NO production is regulated at the transcriptional level by proinflammatory cytokines and endotoxin; and has potent effects on bone resorption (Evans et al. 1996).

Studies have shown that calcium, cGMP, and cAMP act as key mediators or second messengers in the function of many drugs and hormones (Davidovitch, Shanfeld 1975). Intracellular second messengers play an important role in the differentiation of osteoclasts from their precursors (monocytes) in bone resorption during mechanical force application (King et al. 1991). Some studies have shown that low concentrations of NO inhibit osteoclast function and that this action is cGMP dependent (Holliday, Dean, Lin, Greenwald, Gluck 1997). In addition, osteoblast function is governed by a nitric oxide dependent pathway that is mediated by the second messenger cGMP (Mancini et al. 2000). It is well known that the local microenvironment is crucial to the regulation of osteoclast activity; and that a number of factors produced by osteoblasts modulate the proliferation and differentiation of osteoclast precursors and the function of mature cells (Mancini et al. 1998). Nitric oxide is of major importance as one of the local factors that regulates bone metabolism. Nitric oxide is produced by osteoblasts; and inhibits the
function of mature osteoclastic cells (Mancini et al. 1998). Therefore, cytokine-induced NO released from osteoblasts can act to down-regulate osteoclast formation and activity, thereby identifying NO as a potentially important osteoblast-osteoclast coupling factor (Van’T Hof, Ralston 1997).

Nitric oxide has been suggested to have a biphasic effect on bone remodeling. It has been shown that high NO concentrations inhibit resorption by mature osteoclasts and also inhibit the production of osteoclasts from its precursor cells. On the contrary, it has been suggested that low concentrations of NO stimulate osteoblast growth and cytokine production (Collin-Osdoby, Nickols, Osdoby 1995). Recently, however, it has been shown that low concentrations of NO actually inhibit osteoclast formation (Holliday et al., 1997). Thus, the effects of NO on bone remodeling appear to be dependent on dosage and the specific nature of the system. For instance, the role of NO in remodeling long bones may be different from that in calvaria. Therefore, it was not possible to predict the role, if any, on NO in orthodontic tooth movement. To test for the role of NO in OTM, we used the general NO synthase inhibitor L-nitro-arginine-methyl ester (L-NAME).

The formation of nitric oxide can be inhibited by substituting arginine analogues such as L-N-monomethyl arginine (L-NMMA) and L-nitro-arginine-methyl ester (L-NAME) (Figure 1). Our aim was to demonstrate the effect of nitric oxide inhibition on orthodontic tooth movement using L-NAME.

Data from this study provide information regarding the possibility of manipulating OTM by pharmacological intervention of NO signaling. Since agents are attractive target for bone specific pharmaceuticals.
Figure 1. Synthesis and metabolism of NO
CHAPTER 2
MATERIALS AND METHODS

Animals

Experiments were performed according to methods described by King et al. (1991). Two hundred fourteen male Sprague-Dawley rats (20 to 30 days old) were used because of availability, cost, genetic homogeneity, and (at this age) the ability to recover rapidly from the surgeries required to place the appliances. Male rats were chosen to eliminate the hormonal changes associated with estrus. Upon their arrival they were acclimated for 5 to 7 days under experimental conditions.

Orthodontic Treatment

The rats were weighed and profoundly anesthetized using intra-muscular injections of ketamine (87 mg/kg) and xylazine (13 mg/kg). The occlusal surfaces of both maxillary first molars were prepared by roughening the surface and modified orthodontic cleats were bonded. The opposing mandibular first molars were extracted and all four incisors pinned to prevent further eruption. The animals were then allowed to recover for 3 weeks, while monitoring wound healing and weight gain.

Upon their recovery, the rats were randomly divided among four experimental groups:

- Control/drift with drug (CD)
- Control/drift without drug (C)
- Springs with drug (SD)
- Springs without drug (S)
A 0.5 mg/mL concentration of the nitric oxide inhibitor L-NAME was placed in the drinking water for each rat which they received *ad libitum*. Each control group comprised of 5 to 6 rats. Each treated (springs) group comprised 8 to 9 rats. These were sacrificed at 0, 1, 3, 5, 7, and 10 days after orthodontic spring activation.

Appliances were activated by positioning the rats in a head restrainer and orthodontic springs were placed. One end of a length of closed coil spring (7 mm length of Unitek Hi T coil with a wire diameter of 0.006 inches and an inner diameter of 0.022 inches) was ligated to the molar cleat while the other was attached to a 40g suspended weight. This force has been chosen since it has been shown to demonstrate the typical OTM kinetics and acceptable balance between bone formation and resorption (King et al. 1991). The anterior end of the coil was then bonded with autocuring methacrylate to the acid-etched lateral surface of the maxillary incisors, followed by removal of the weight and excess coil spring. This method insured a precise and reproducible initial orthodontic force designed to tip the maxillary first molars to the mesial and to yield appliances with equivalent decay rates. The strain environments created are primarily compression on the mesial surface and tension on the distal. The control group received all the procedures done except spring placement.

**Radiographic Examination**

Rats were placed in the headholder and radiographed at the placement of the appliances and at the time of sacrifice. These cephalograms routinely display the endodontic implants, molar cleat and orthodontic springs and were utilized to calculate tooth movement in each animal. A total of four cephalograms were taken for each rat for each time period in all groups. This was done to so that an average value could be calculated. The 95% confidence limit for the molar-to-broach method could be reduced
to 23 µm when the average of four independent determinations was used (King et al. 1991). The cephalograms were then digitized at 600 dpi and analyzed using the NIH imaging program ImageJ. OTM was measured from the molar cleat to the distal pin on the incisor. The difference between these measurements made on appliance placement and radiographs taken at sacrifice was calculated and reported as tooth movement (King et al. 1991).

**Histological Examination**

In order to study the histological changes in bone and the tissues surrounding the tooth, the posterior portion of the maxilla containing teeth, bone and tissue was dissected and fixed in a paraformaldehyde fixative solution (Shirazi, Nilforoushan, Alghasi, Dehpour 2002). The soft tissue was then removed and the hard tissue was placed in a demineralization solution. Thereafter, the bone was dehydrated in ethanol and xylene and then infiltrated with paraffin and xylene prior to being embedded in paraffin. 4µm thick samples were treated with 0.1% pepsin for enzymatic digestion that allowed for better solution infiltration and embedded in paraffin. A series of antibodies to NOS (Oncogene Research Products) were used to bind to the exposed epitope on the tissue samples. They were finally stained with 3,3’ Diaminobenzidine tetrahydrochloride (DAB). The intensity of staining of the PDL around the distal root of the maxillary first molar was the criteria for comparison among the samples.

**Statistical Analysis**

The means and standard errors of molar movement were calculated for each time point. Statistical evaluation of the data was done with the analysis of variance
(ANOVA). The paired $t$ test was used to evaluate the significance of difference in tooth movement between the rats treated with and without study drug (L-NAME).
CHAPTER 3
RESULTS

The body weight of all of the rats in the four groups was recorded for three different time points: surgery, activation and sacrifice. This was done to observe any irregular eating habits due to trauma caused by surgery, activation of springs or the effect of L-NAME (Figure 2) on body weight. The weight of the rats increased substantially after surgery in all 4 groups. However it is evident that the rats may have had some discomfort during food consumption due to intraoral appliances since their body weight decreased slightly from the day of springs activation to sacrifice (Figures 2 and 3).

The amount of L-NAME consumption was noted for both the molar drift and springs groups. Both groups consumed approximately the same amount of NOS inhibitor (L-NAME) (Figure 4).

As a note, the data for Day 1 of OTM with L-NAME are missing and therefore not included in Figure 5 or the discussion to follow. Tooth movement in the OTM groups exhibited the three typical phases of tooth movement. An initial compression of the periodontal ligament accounts for the 0.4 mm of molar movement from Day 0 to 1. A lag phase with no tooth movement was noted thereafter (from Day 1 to 5). This is the period where necrotic tissue accumulated in the compressed area of the PDL is eliminated. Finally, orthodontic tooth movement occurs from Day 5 to 10. The OTM group with L-NAME showed similar tooth movements as the OTM control group until Day 5. Throughout the period of Day 5 to 10 the OTM with L-NAME group showed remarkably less tooth movement than the control. On the final day it was noted that molar movement
in the OTM with L-NAME group was approximately 0.2 mm less than that of the OTM control group and was statistically significant at P<0.0001.

L-NAME caused a significant reduction in tooth movement in the distal drift groups (after Day 3) as compared to the orthodontically treated groups (after Day 5). Perhaps the orthodontic appliances overpowered the effects of the NOS inhibitors on the biological systems of the rats.

The distal drift control and distal drift with L-NAME groups showed some dissimilarities (Figure 6). From activation to Day 2, the distal drift with L-NAME group showed more molar movement than the control group. After Day 2 until Day 10, the distal drift control group showed more molar movement than the distal drift with L-NAME group. The difference in tooth movement between the distal drift and the drift with L-NAME groups was statistically significant for Days 5 (P<0.0002) and 7 (P<0.001). On the final day it was noted that molar movement in the distal drift with L-NAME group was approximately 0.3 mm less than that of the distal drift control group and was statistically significant at P<0.022.

The periodontal ligament space around the distal root of the maxillary first molar was carefully examined for the intensity of DAB staining that occurs as an indication of NOS expression. The results show less intensity of staining around the roots of those rats treated with L-NAME (Figure 7) as compared to control rats. These findings are consistent with our hypothesis that L-NAME would serve to block the enzymatic activity of nitric oxide synthase in rat specimens. In addition, there was no difference in gross morphology of the roots between L-NAME treated rats and control rats.
Figure 2. Change in body weight (gm) of rats receiving L-NAME at three distinct time points: surgery, activation and sacrifice.

Figure 3. Change in body weight (gm) of rats not receiving L-NAME at three distinct time points: surgery, activation and sacrifice.

Figure 4. Consumption of L-NAME (ml) by the control and springs groups.

Figure 5. Orthodontic tooth movement from Day 1-10 of spring rats vs. spring rats receiving the NOS inhibitor.
Figure 6. Orthodontic tooth movement from Day 1-10 of molar drift rats vs. molar drift rats receiving the NOS inhibitor

Figure 7. Photomicrographs showing the distal root of the maxillary first molar. A) From a rat treated with L-NAME. B) From a control rat. Slides were stained with DAB
The results of the present study highly suggest that nitric oxide (NO) has a significant modulatory effect on tooth movement. Systemic administration of L-NAME, a nitric oxide synthase inhibitor, in rats led to a decrease in orthodontic tooth movement and distal molar drift compared to controls. Thus, it appears that nitric oxide production is essential for an optimal response by the periodontal ligament upon application of orthodontic forces in the rat model.

The present results clearly demonstrate that the systemic administration of L-NAME caused a significant reduction in tooth movement in the treated and control/drift groups. All groups showed similar rates of tooth movement from day 0 to day 1, however, in the final phase of bone remodeling from day 3 to day 10, the L-NAME treated groups exhibited less tooth movement than the OTM/drift control groups (Figures 5 and 6).

Fourteen rats died among the L-NAME treated groups whereas only 2 died in the non-drug treated groups. Rats may suffer from high blood pressure secondary to systemic L-NAME administration (Tucker, Ledingham, Zheng, Laverty 2000). Since nitric oxide (NO) is a signaling molecule in the cardiovascular, immune and nervous systems, it has a wide range of effects throughout the body. Nitric oxide influences blood pressure not only via vascular endothelial release, but also by central cardiovascular control. Hypertension is a multifactorial disease that may be defined as a sustained elevation in blood pressure resulting in target organ damage. In essential hypertension,
the majority of evidence indicates that there is an abnormality in the L-arginine/NO pathway (Tucker et al. 2000). Some studies have shown that general NOS inhibition elicited a sustained blood pressure increase, a decrease in heart rate, cardiac hypertrophy and an increase in wall thickness of the coronary and carotid artery (Gerova 2000). It is quite possible that the increase in blood pressure among the rats in all four groups may have affected the results of the study since they had systemic complications secondary to L-NAME administration. Therefore, the tooth movement effects noted may not be due to NOS inhibition and strictly a consequence of the effects on the biological system induced by high blood pressure. In future studies, a more local administration of the NOS inhibitor or a reduced concentration may prevent these untoward effects.

Currently, an applicable approach for local delivery of the NOS inhibitor L-NAME is through Elvax 40. This is a non-biodegradable, noninflammatory sustained release polymer that has been used as a vector to deliver pharmacologic agents to a precise location. Elvax would serve to aid in the elimination of the adverse systemic effects of nitric oxide that have been documented.

Perhaps locating a cNOS inhibitor would be more effective since we would not be affecting iNOS. It would also be beneficial to investigate the effects of nitric oxide using a NOS stimulus (e.g. sodium nitroprusside) as opposed to a NOS inhibitor as was done in the present study. This would prevent the increase in blood pressure since nitric oxide production would not be attenuated. However, it may also serve to lower blood pressure which could also be a problem. The application of a phosphodiesterase V inhibitor (e.g. Zaprinest, Sildenafil) may be used in order to prevent the breakdown of cGMP. Therefore, cGMP levels are higher as though there is more NO present (Figure 1).
Recently, other researchers have demonstrated the effects of the involvement of nitric oxide in orthodontic tooth movement in rats. Hayashi et al. noted results similar to those of the present study. Our results confirm their findings that the administration of L-NAME, a nitric oxide synthase inhibitor, significantly reduced tooth movement. However, they found that the local administration of L-NIL (N-1-iminoethyl-L-lysine), a selective inhibitor of iNOS, did not affect orthodontic tooth movement (Hayashi 2002).

In conclusion, our results indicate that L-NAME decreases orthodontic tooth movement compared to control subjects. This is an exciting finding in that it may be of great use in the field of orthodontics. Local application of L-NAME and its subsequent effects on tooth movement may allow the clinician greater control of orthodontic forces. These results suggest that nitric oxide is instrumental in orthodontic tooth movement and our study has shown that blocking NOS systemically significantly reduces tooth movement. However, concerns that hypertension resulting from systemic NOS inhibition must be addressed. If NOS inhibitors prove to be specifically blocking OTM, they may be advantageous in regards to anchorage control and, more importantly, the management of relapse. In the future, NOS inhibitors may prove to serve as a pharmacological means of providing anchorage for clinicians. Administering L-NAME locally may allow the orthodontist to gain full control of the dentition in specific areas during tooth movement thereby eliminating many appliances needed for anchorage control. Moreover, it may be of benefit in preventing a relapse of tooth movement and permit a more sustained orthodontic result.
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BIOGRAPHICAL SKETCH

Arvind Kenneth Vakani was born in New York and raised in South Florida. He attended both Rollins College and the University of South Florida for his undergraduate studies; and then the University of Florida for his dental education. Dr. Vakani graduated with High Honors from the University of Florida in 2000, obtaining a Doctor of Dental Medicine degree. After graduation, Dr. Vakani furthered his dental education at the University of Florida to complete a degree of Master of Science with a certificate in orthodontics.