

# Gingival Endothelial and Inducible Nitric Oxide Synthase Levels During Orthodontic Treatment: A Cross-Sectional Study

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**Abstract:** This study uses a cross-sectional design to examine the endothelial and inducible nitric oxide synthase (eNOS and iNOS, respectively) levels of gingival tissue. Fifteen subjects, 10 female and 5 male individuals (aged 14.6–21.2 years; mean  $17.4 \pm 1.8$  years), who needed extraction of the four first premolars for orthodontic reasons and who had indications for a gingivectomy were enrolled in the study. In each patient, two maxillary/mandibular premolars were extracted, and two months later an orthodontic appliance was placed in the same arch. A canine undergoing treatment for distal movement served as the test tooth (TT), whereas its contralateral canine was used as the control tooth (CT). The CT was included in the orthodontic appliance but was not subjected to the orthodontic force. Two weeks after the orthodontic appliance placement, clinical data consisting of the presence of supragingival plaque, bleeding on probing, and probing depth were collected from each experimental tooth. Immediately after, gingival tissue was collected from the distal aspect of each TT and CT for immunohistochemistry, messenger RNA reverse transcription by polymerase chain reaction, and Western blot analysis for both eNOS and iNOS. The results showed that no differences in clinical conditions occurred between the experimental teeth. On the contrary, both the eNOS and iNOS levels and the expression of the TTs were significantly greater than those of the CTs (all comparisons significant to  $P < .01$ ). Our results indicate a role for gingival eNOS and iNOS during the early phases of orthodontic treatment in humans. (*Angle Orthod* 2004;74:851–858.)

**Key Words:** Orthodontic tooth movement; Nitric oxide synthase; Tissue remodeling/inflammation

## INTRODUCTION

Tooth movement induced by orthodontic forces is consequent to remodeling of the periodontal tissues.<sup>1–3</sup> There have been studies that have evaluated the hard- and soft-tissue responses as biological features during orthodontic treatment in animal<sup>4–7</sup> as well as in human<sup>8,9</sup> models. How-

ever, most of these have focused their attention on bone metabolism<sup>5,6</sup> or periodontal ligament (PDL) changes<sup>2–4</sup> that occur during tooth movement. Gingival modifications incidental to tooth movement have been reported as occurring in both histological and ultrastructural analyses and at a clinical level in certain cases.<sup>10</sup>

Nitric oxide (NO) is a short-lived, highly reactive free radical involved in a variety of cellular pathways. Indeed NO is an intercellular messenger molecule released from various host cells and has important cardiovascular, neurological, and immune functions.<sup>11</sup> NO released from osteoclasts is also involved in bone resorption.<sup>12</sup> The synthesis of NO is catalyzed by the NO synthase (NOS) family of enzymes that contain three mammalian gene products. NOS isoforms are functionally distinguished by their modalities of regulation. Two Ca<sup>2+</sup>-dependent isoforms of NOS, neuronal (nNOS) and endothelial (eNOS), remain dormant until Ca<sup>2+</sup>-calmodulin binding is elicited by a transient elevation in the intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]). In contrast, Ca<sup>2+</sup>-independent NOS (iNOS) is constitutively active, even at low resting levels of intracellular [Ca<sup>2+</sup>], because of a remarkably high affinity for calmodulin. The three isoforms are further differentiated by their maximal

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rates of NO synthesis; nNOS and iNOS exhibit several-fold greater activities than eNOS.<sup>11</sup> Moreover, iNOS is able to produce NO for a longer period of time than either eNOS or nNOS, both of which produce NO in a pulsatile fashion.<sup>11</sup> Finally, both eNOS and nNOS are ubiquitously present in tissues, whereas iNOS is detectable only in inflamed tissues.<sup>11</sup>

The presence of eNOS and iNOS has been shown in dental tissues.<sup>13,14</sup> Moreover, increased levels of gingival iNOS during periodontal inflammation have been reported, as compared with noninflamed gingival tissue.<sup>15,16</sup> Indeed, although iNOS is not ubiquitously present in tissues,<sup>11</sup> immunohistochemistry analyses<sup>15,16</sup> have revealed that clinically healthy gingival tissue has detectable amounts of iNOS. Evidence of the expression of iNOS in epithelial<sup>17</sup> and inflammatory<sup>18</sup> cells of the gingiva has also been seen. Other *in vitro* investigations<sup>19,20</sup> have reported noteworthy changes in NO levels in PDL fibroblasts when they are mechanically stressed.

To date, studies have neither shown the localization of eNOS in human gingival tissue nor investigated the possible role of gingival iNOS in the early events of orthodontic treatment. Thus, the present study was designed as a cross-sectional assessment to determine whether eNOS levels are detectable in gingival tissue and whether eNOS and iNOS are sensitive markers for gingival tissue changes during the early phases of such treatment in humans.

## MATERIALS AND METHODS

### Study population

Fifteen patients, 10 female and 5 male individuals (aged: 14.6–21.2 years; mean  $17.4 \pm 1.8$  years), who had presented at the Unit of Orthodontics, Department of Oral Sciences, University G. D'Annunzio (Chieti) and who were diagnosed for the extraction of their first premolars because of dental crowding participated in this study. The following inclusion criteria were observed: (1) the need for fixed-appliance therapy involving distal retraction of one maxillary/mandibular canine, with an indication for gingivectomy to overcome tooth relapse after closure of the extraction sites,<sup>21</sup> (2) a healthy systemic condition, (3) no use of anti-inflammatory drugs in the month preceding the beginning of the study, (4) probing depth values (measured as the distance from the bottom of the sulcus to the most apical portion of the gingival margin) not exceeding four mm in the whole dentition, (5) no loss of periodontal attachment (measured as the distance from the bottom of the sulcus to the cemento-enamel junction) exceeding two mm in any interproximal site, (6) no radiographic evidence of periodontal bone loss after a full-mouth radiographic periapical examination, and (7) a full-mouth plaque score (FMPS) and a full-mouth bleeding score (FMBS)  $\leq 20\%$ . FMPS and FMBS were recorded as the percentage of tooth surfaces with the presence of supragingival plaque or bleeding with-



**FIGURE 1.** Illustration of the full orthodontic appliance used in the present study.

in 15 seconds of probing with a 20-g controlled-force probe. Informed consent was obtained from the patients and the parents of patients under 18 years of age before the commencement of the study, and the protocol was reviewed and approved by the Ethical Committee of the Medical Faculty of the University G. D'Annunzio.

### Clinical procedures

Two maxillary/mandibular first premolars were extracted for each participant, and during the following two months, all subjects received repeated oral hygiene instructions (OHIs), which included the correct use of a toothbrush, dental floss, and an interdental brush. At the end of this period, one maxillary/mandibular canine (of the dental arch corresponding to the extracted premolars) was distalized and considered as the test tooth (TT), whereas the contralateral canine was used as the control tooth (CT). On the corresponding arch of the TT of each subject, orthodontic brackets (MBT, 3M-Unitek, Monrovia, Calif) were placed on the buccal surfaces of the incisors, canines, and premolars, and bands were placed on the first molars. A unilateral 0.018-inch nickel titanium (MBT) arch wire was then fixed from the central incisor to the first molar in the same quadrant as the TT. At the same time, a nickel titanium open coil spring (American Orthodontics, Sheboygan, Wis), exerting a constant force of 150 g, was included in the appliance to move the TT distally.<sup>22</sup> Finally, the central and lateral incisors in the same quadrant as the TT were laced together with a continuous 0.010-inch steel orthodontic wire to provide anchorage. The entire orthodontic appliance (Figure 1) was placed in a single clinical session.

Further OHIs were given to the subjects on how to perform effective tooth cleaning in the presence of the orthodontic appliance, and they were not allowed to take anti-inflammatory drugs for the entire duration of the study. Moreover, all patients underwent one session of accurate supra- and subgingival ultrasonic scaling two weeks before the orthodontic appliance placement.

Two weeks after the orthodontic appliance placement, six sites (mesio-, mid-, and distobuccal; mesio-, mid- and distolingual/palatal sites) on the test and control teeth were clinically examined for the presence of (1) supragingival plaque (PL+), assessed by visual criteria; (2) gingival bleeding within 15 seconds of probing (BOP+) with a 20-g controlled-force probe; and (3) probing depth (PD). The same operator always collected the clinical data (MD'A).

Immediately after completion of the clinical data, gingival tissue distal to both the TTs and the CTs was collected under local anesthesia for eNOS and iNOS analyses. The tissue was transferred to plastic vials and immediately stored at  $-80^{\circ}\text{C}$  for 48 hours. Subsequently, multiple seven- $\mu\text{m}$  tissue slices were obtained using a cryostat (Reichert-Jung Frigocut 2800, Monochengladback, Germany). These slices were placed on slides, previously treated with gelatin and chromium alum, and kept at a constant temperature of  $20^{\circ}\text{C}$ . Randomly selected slices from each group were stained with hematoxylin-eosin for histopathological analysis.

### Immunohistochemistry for eNOS and iNOS

The anti-eNOS and -iNOS immunohistochemical reactions were performed at room temperature in a humid chamber. The defrosted sections were rinsed with Tris-HCl buffer (pH 7.4) for five minutes in a petri dish. After blocking the nonspecific reactivity with 3% goat serum (Santa Cruz Biotech Inc., Santa Cruz, Calif) for 30 minutes in Tris-HCl buffer, the sections were again rinsed with Tris-HCl buffer (three  $\times$  five minutes) and incubated with primary rabbit anti-human eNOS or iNOS polyclonal antibodies (Santa Cruz Biotech Inc.), at a dilution of 1:100 in phosphate-buffered saline (PBS), for 30 minutes at room temperature. Subsequently, the sections were washed with Tris-HCl buffer (two  $\times$  five minutes) and incubated with a secondary goat anti-rabbit antibody (Santa Cruz Biotech Inc.), at a dilution of 1:100 in PBS, for 30 minutes at room temperature. They were then washed with Tris-HCl buffer (three  $\times$  five minutes) and incubated with the peroxidase-antiperoxidase complex (Santa Cruz Biotech Inc.) for 10 minutes. The sections were then washed with Tris-HCl buffer (two  $\times$  five minutes), and the peroxidase was developed using diaminobenzidine, a chromogen (Santa Cruz Biotech Inc.), mixed in an imidazole buffer (pH 7.6) for 10 minutes. The sections were then dehydrated, clarified, and mounted with a Permount. The presence of eNOS or iNOS was identified by the appearance of a brick red precipitate in the gingival structure.<sup>23</sup> Immunohistochemical specificity was tested by omitting the antisera before immunohistochemical staining.

### Reverse transcription by polymerase chain reaction for eNOS and iNOS messenger RNA

Total RNA was extracted using one mL RNazol (Cinna Biotex, Huston, Tex) with 20  $\mu\text{g}$  *Escherichia coli* ribosomal

RNA (rRNA) (Boehringer, Ingelheim GmbH, Germany) as carrier. Reverse transcription (RT) was performed in a volume of 20  $\mu\text{L}$  containing M-MLV reverse transcriptase (Perkin-Elmer, Wellesley, Mass), one mM deoxynucleoside triphosphate, 2.5  $\mu\text{M}$  random primers, and one unit/ $\mu\text{L}$  RNase inhibitor (Pharmacia Corp., North Peapack, NJ) for 30 minutes at  $42^{\circ}\text{C}$ . Polymerase chain reaction (PCR) amplification was performed using an Eppendorf Mastercycler 5330, operating at a  $72^{\circ}\text{C}$  step temperature of 60-seconds length. The  $\text{MgCl}_2$  concentration used for human eNOS and iNOS complementary DNA amplification was 2.0 mM. The following primer pairs were used: 5'-TGTCTGTCTGCTGCTAG-3' (sense) and 5'-CTCTCCAGGCACTTCAGGC-3' (antisense) for human eNOS and 5'-AGTGATGGC AAGCACGACTTC-3' (sense) and 5'-TCTGTCACTCGC TCACCACGG-3' (antisense) for human iNOS. A PCR was performed using 18S rRNA as internal standard as previously described.<sup>24</sup>

### Western blot analysis for eNOS and iNOS

After homogenizing the gingival tissues in lysis buffer (10% PBS, 10% NP-40, 10% sodium deoxycholate, 10% sodium dodecyl sulfate [SDS], and a protease inhibitor cocktail containing aprotinin, leupeptin, and  $\text{Na}_3\text{VO}_4$ ) (Pharmacia Corp.), equal amounts of protein (50  $\mu\text{g}$ ) from the gingival specimens were separated by electrophoresis in a 7.5% polyacrylamide-SDS gel (BioRad, Hercules, Calif) and transferred at  $4^{\circ}\text{C}$  to nitrocellulose membranes (BioRad) in glycine-methanol buffer.  $\beta$ -Actin was also run as an internal standard of 130 kDa. The nitrocellulose was then blocked in Tris-buffered saline (TBS)-milk and incubated overnight with the primary anti-eNOS or -iNOS antibodies (BioRad). The nitrocellulose was washed in TBS, incubated with the secondary antibodies conjugated with alkaline phosphatase for two hours, rewash, and developed in an alkaline buffer with NBT (alkaline phosphatase conjugate substrate kit) (BioRad).<sup>14</sup>

### Image processing and analysis

The immunohistochemical sections of the gingival specimens were examined under a Leitz Dialux 22 microscope (Leica, Heidelberg, Germany). The quantitative evaluation of the eNOS and iNOS immune reactions and the messenger RNA (mRNA) and protein levels was performed by determination of the integrated optical density (IOD) scores by digital image analysis.<sup>14</sup> The areas investigated for immunohistochemistry were randomly chosen and recorded from five slides in each group. For data processing, each experimental frame was digitized into  $512 \times 512$  pixels by a Sony video camera connected to a Leica Quantimet 500 plus microscope, and the IOD scores, from 1 to 4 according to the increasing optical density, were determined using ISO Transmission Density (Kodak CAT 152-3406, Eastman Kodak Company, Rochester, New York) as a standard.

### Data processing

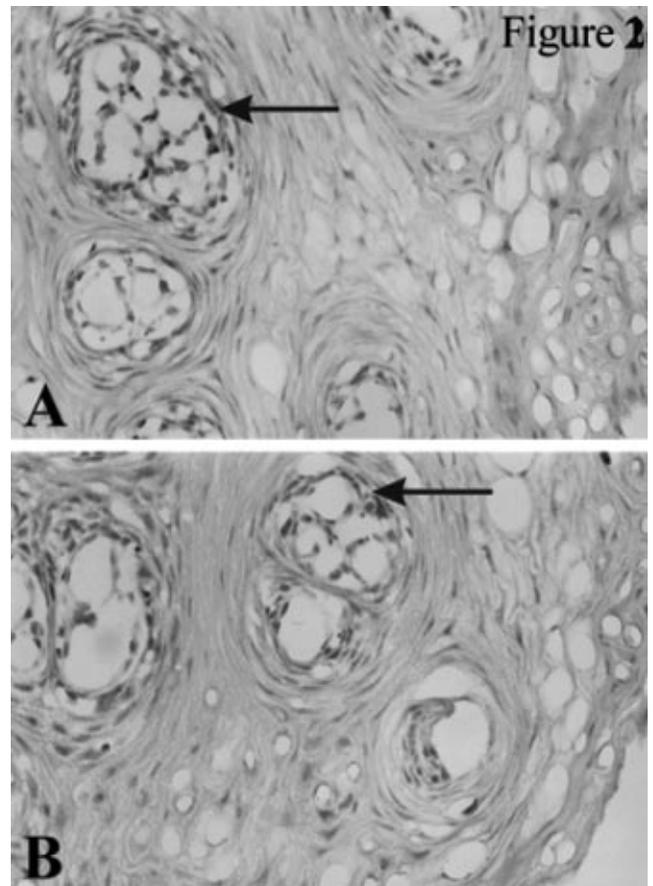
The Statistical Package for Social Sciences software (SPSS® Inc., Chicago, Ill) was used to perform the data analysis. The percentage of tooth sites positive for plaque (%PL+), bleeding on probing (%BOP+), and mean PD were calculated for the TT and CT groups, considering the tooth as the statistical unit. Wilcoxon paired signed rank tests were used to evaluate the statistical significance of the differences in the %PL+ and %BOP+ between the experimental categories. A paired *t*-test was used to assess the significance of the differences in the PD between the experimental groups. Moreover, differences in the clinical data between the TTs and CTs obtained from the corresponding experimental collection sites were also tested. The number of PL+ and BOP+ experimental sites were processed as paired dichotomous data by using a McNemar test, whereas the PD scores were assessed performing a paired *t*-test.

For both the eNOS and iNOS immune reactions and the mRNA and protein levels, the Wilcoxon paired sign rank sum test was used to evaluate the significance of the differences in the IOD scores between the experimental groups. A probability of  $P < .05$  was accepted for rejection of the null hypothesis.

### RESULTS

The %PL+, %BOP+, and mean PD in the TTs and CTs were similar, with no statistically significant differences seen between the groups (all significance levels at  $P > .5$ ; data not shown). Similarly, no significant differences were detected between the TTs and CTs in all the clinical parameters of the corresponding collection sites ( $P > .5$ ; data not shown). Finally, pooled data regarding %PL+, %BOP+, and mean PD from the TTs and CTs, presented as means  $\pm$  standard errors, are  $18.4 \pm 2.5$ ,  $12.4 \pm 1.9$ , and  $1.9 \pm 0.1$ , respectively.

Epithelial cells, connective tissue, blood vessels, nerve fibers, and lymphocytes could all be detected in all the histological sections from both the TT and the CT gingival specimens. An overall impression through the analysis of the full range of samples suggests that vasodilatation and leukocyte infiltration (predominantly macrophages and plasma cells) appeared to be more evident in the TT gingival specimens than in those of the CTs (data not shown). However, representative immunohistochemical sections of gingival specimens from both the TT and the CT groups, illustrating the positive precipitates for eNOS and iNOS, are shown in Figures 2 and 3. All the specimens examined were positive for the presence of both these NOSs. Moreover, endothelial cells and fibroblasts were predominantly positive for eNOS (Figure 2), whereas iNOS immunoreactivity was found mainly along the basal membrane and in inflammatory cells within the connective tissue (Figure 3).



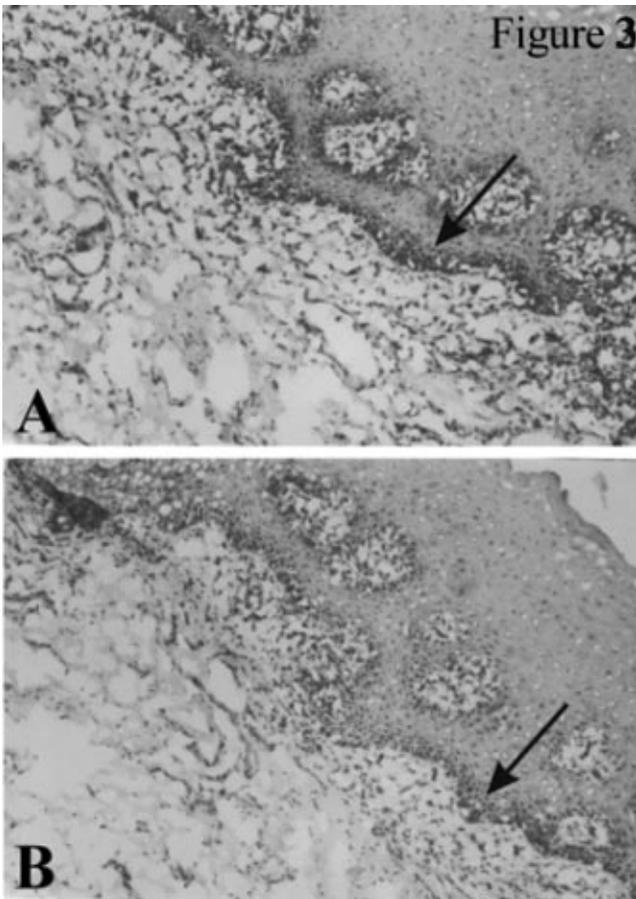
**FIGURE 2.** Immunohistochemical localization of eNOS in the (A) TT and (B) CT groups (magnification 250 $\times$ ). Endothelial cells and fibroblasts are predominantly positive for eNOS (arrows). eNOS indicates endothelial nitric oxide synthase; TT, test tooth; and CT, control tooth.

The results regarding the NOSs' immune reactions and the mRNA and protein levels in each TT and CT group are summarized in Table 1. In particular, the IOD scores of both eNOS and iNOS were higher in the TTs than in the CTs in the immunohistochemistry (Figures 2 and 3), RT-PCR (Figure 4), and Western blot (Figure 5) investigations.

### DISCUSSION

Our controlled cross-sectional study evaluated enzymatic gingival changes that occur during the early phases of orthodontic treatment in humans. Although no differences were detected in the clinical conditions, the eNOS and iNOS immunoreactivities and the mRNA and protein levels were significantly greater in the gingival tissues compressed by the tooth movement (TTs) than in those of the untreated controls (CTs) (Table 1).

Although several experimental models have been used to evaluate periodontal and dental tissue responses to mechanical stimuli,<sup>7</sup> only a limited number of studies have been carried out on human subjects, and most of the data avail-



**FIGURE 3.** Immunohistochemical localization of iNOS in the (A) TT and (B) CT groups (magnification 100×). Basal membranes and inflammatory cells within the connective tissue are predominantly positive for iNOS (arrows). iNOS indicates inducible nitric oxide synthase; TT, test tooth; and CT, control tooth.

able have been obtained from animal models.<sup>7</sup> In vitro experiments have been performed on human periodontal cells under mechanical stress,<sup>8,19</sup> but such a study design cannot account for the complexities of the in vivo situation. Indeed, orthodontic forces can affect all periodontal and dental tissues, and the pattern of reaction of one has been seen related to the responses in the other.<sup>7</sup> The present study has the advantage of evaluating the gingival response to compression stress using an in vivo design where the complexity of the interactions among the periodontal tissues has been accounted for. Moreover, the use of a control within the same patient is effective in controlling the individual patient responses.

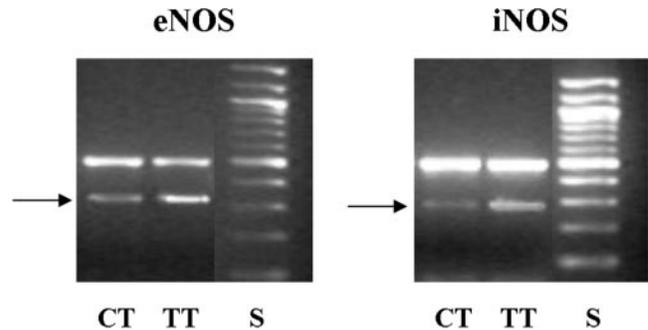
The study population enrolled in the present investigation showed similar gingival conditions for the TTs and CTs, probably because of the repeated OHIs imparted to each participant before the beginning of the treatment. Moreover, it has been reported that good maintenance of clinical conditions is possible despite orthodontic appliance placement.<sup>25</sup> The short duration of the study design also may have contributed to these results.

**TABLE 1.** Distribution of IOD scores and medians for eNOS and iNOS immunoreactivities, and mRNA and protein levels in the experimental groups (n = 15).

		Group	Number of Specimens in Each IOD Score				Median IOD Score
			1	2	3	4	
eNOS	Imm.*	TT	1	4	6	4	3
		CT	7	6	2	0	2
	RT-PCR*	TT	0	3	8	4	3
		CT	8	7	0	0	1
	WB*	TT	0	4	8	3	3
		CT	10	3	2	0	1
iNOS	Imm.*	TT	1	3	4	7	3
		CT	11	3	0	1	1
	RT-PCR*	TT	2	2	6	5	3
		CT	11	2	2	0	1
	WB*	TT	0	4	6	5	3
		CT	13	1	1	0	1

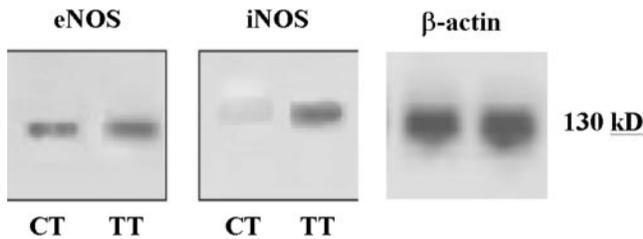
<sup>a</sup> TT indicates test tooth; CT, control tooth; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; IOD, integrated optical density; Imm., immunohistochemistry; RT-PCR, reverse transcription by polymerase chain reaction; and WB, Western blot analysis.

\* Level of significance of the differences between the TT and CT groups, within either eNOS or iNOS, by analytical investigation: \*p < .01.



**FIGURE 4.** Reverse transcription by polymerase chain reaction for eNOS and iNOS mRNA in the TT and CT groups (arrows). The last lane shows the standard (S). Higher mRNA expression is evident for both eNOS and iNOS in the TT group as compared with the CT group. eNOS indicates endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; mRNA, messenger RNA; TT, test tooth; and CT, control tooth.

All the histological samples obtained in the present study showed an inflammatory infiltrate, with an overall impression that vasodilatation and leukocyte infiltration (predominantly macrophages and plasma cells) appeared to be more evident in the TT, as opposed to the CT, gingival specimens. Because it is virtually impossible to obtain pristine healthy tissue samples from humans despite the absence of any clinically detectable signs of inflammation, and because these apparent differences in the TTs as opposed to the CTs were both subtle and not consistently seen, this aspect requires further specific investigation. However, all the ana-



**FIGURE 5.** Western blot analysis for eNOS and iNOS in the TT and CT groups.  $\beta$ -Actin was used as an internal standard (130 kDa). Higher levels are evident for both eNOS and iNOS in the TT group as compared with the CT group. eNOS indicates endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; TT, test tooth; and CT, control tooth.

lytical investigations demonstrated detectable levels and expression of NOSs in both the TT and the CT gingival tissues.

High levels of eNOS have been seen in the endothelial cells of the blood vessels,<sup>26</sup> and it was not surprising to find detectable immunoreactivity of eNOS in the endothelial cells of the gingival tissues from both the TT and the CT groups (Figure 2). The NO produced by these cells is responsible for the relaxation of the smooth muscle and, hence, the blood flow to the tissues.<sup>26</sup> Previous studies have demonstrated that the NO involved in the maintenance of resting blood flow in dental tissues derives from both eNOS and nNOS activities.<sup>13</sup>

During orthodontic tooth movement, animal research has described an intense vascular activity in the PDL and alveolar bone that decreases one day after the orthodontic force is applied, which increases after three days.<sup>27</sup> Similar results have been obtained in other investigations, which have also reported a remarkable dilatation of blood vessels corresponding to the areas compressed by the tooth movement.<sup>2,28,29</sup> These studies evaluated the vascular changes in the PDL and alveolar bone only and not in the gingival tissues. However, considering that the pattern of reactions in any of the periodontal tissues is strongly related to those of the other compartments,<sup>7</sup> the increase in blood flow consequent to an increase in blood vessel diameter might also occur in the gingival tissue. This hypothesis is supported by the increase in eNOS levels found in the TT gingival tissues as compared with those of the CT group.

A correlation between iNOS levels and inflammation in periodontal tissues has been demonstrated previously.<sup>15,16</sup> However, we can be certain that the higher iNOS levels from the TTs, in comparison with the CTs, were not due to inflammation because all the experimental sites showed good clinical conditions throughout the duration of the study. However, during orthodontic tooth movement in humans, an increase in several inflammatory mediators in the gingival crevicular fluid (GCF), ie, interleukins, has been found in spite of a good clinical condition.<sup>30</sup> Hence, these authors concluded that subclinical inflammation may occur as one of the earliest phenomena responsive to the tissue

remodeling. Moreover, cytokines have been shown to stimulate iNOS production from several cells.<sup>11,26</sup> This supports the increase in iNOS levels in the TT gingival tissue because it may be a consequence of an increase in the inflammatory mediators in the periodontal tissues before they migrate to the gingival crevice where they are detected.<sup>31</sup> This hypothesis is reinforced by the evidence that constituents of GCF derive from a variety of sources, including the gingival tissue.<sup>31</sup> Furthermore, inflammatory changes detectable at a histological level have been demonstrated to also directly affect the gingival tissue surrounding orthodontically moved teeth.<sup>7</sup>

Other explanations regarding the increase in both iNOS and eNOS levels and expression in the TT gingival tissues can be derived from a consideration of the functions of their product, NO. Some years ago, the role of NO in relaxation of arteriole wall smooth muscle and in inhibition of platelet aggregation was demonstrated.<sup>32</sup> It is also of interest that there is evidence that NO plays a role in bone resorption,<sup>12</sup> particularly because it is well known that during orthodontic tooth movement bone resorption is constantly present in the sites of compression<sup>2</sup> and even in the sites of tension.<sup>5</sup> Thus, considering the existence of tight anastomoses in the blood vessels between the periodontal tissues,<sup>27,33,34</sup> the increase in NOS levels and expression in the TT gingival tissues two weeks after the orthodontic force was applied might be further explained by the influence of bone activity on gingival composition.

During orthodontic tooth movement, a hyalinization of the most compressed area of the PDL that is induced by the compressive forces has been reported.<sup>2,35</sup> This hyaline zone is described as an area of focal aseptic necrosis that is resistant to degradation, persists in the pressure zone, and is dependent on the magnitude of the force.<sup>35</sup> Other investigations<sup>36,37</sup> have reported increases in the number and activity of macrophage-like cells in the resorptive area of periodontal tissues undergoing mechanical stress, corresponding to the hyaline zone, and this activity is involved in the removal of the necrotic periodontal tissue. Considering further that during orthodontic tooth movement an intense macrophage activity occurs consistently near blood vessels of the periodontal tissues both in the areas of tension and in the areas of resorption,<sup>28</sup> and that NO is also released from macrophages during cell-cell interactions,<sup>32</sup> the increase in the NOS levels and in their expression found in the TT gingival tissues might be due, in part, to the activity of these cells. This hypothesis is reinforced by a recent investigation that showed detectable levels of iNOS in macrophages of gingival tissue.<sup>18</sup>

Further consideration of the results of the present study indicates that they can be linked to previous investigations that have reported increases in NO production from PDL fibroblasts when they are mechanically stressed.<sup>19,20</sup> Although the present study evaluated the NOS levels and expression in gingival tissue, rather than in the PDL, it is

likely that the anatomical and functional connections among the periodontal tissues<sup>27,33,34</sup> contribute, at least in part, to the increases in NOS levels and expression found in the TTs. Moreover, fibroblasts are the most abundant cell type in gingival tissue, and their volume comprises 5.6% of the total gingival volume.<sup>38</sup> It is of interest that human fibroblasts increase their NO basal production under mechanical stimulation and that gingival tissue surrounding a moving tooth does not undergo resorption but is compressed and consequently retracted.<sup>10</sup> In connection with this, a direct increase in NO production, through increases in NOS levels and expression, by the fibroblasts of the compressed gingiva could have occurred in the TT gingival tissues.

Conversely, in the CT gingival tissues where no orthodontic force was applied, both NOS levels and their expression are lower than those of the TT gingival tissues (although always detectable) in all analytical investigations (Table 1). Moreover, the presence of detectable levels of iNOS in normal PDL cells and gingival tissues, and eNOS in clinically healthy pulp tissue has been reported in other investigations.<sup>14–16,19</sup> Hence, this study demonstrates gingival localization of eNOS in human subjects and that both eNOS and iNOS of the gingival tissues can be considered suitable markers for orthodontic treatment in humans, although further investigations are needed to fully elucidate these aspects.

Briefly, these results suggest roles for eNOS and iNOS in gingival tissues undergoing compression stress. Moreover, the increase in their levels and expression may be due to the activity of several cell types that are directly or indirectly involved in the tissue response to mechanical stress.

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