Effects of Doxycycline and Corticotomy on Interleukin-1β Expression during Orthodontic Tooth Movement

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끝없는 이해와 사랑을 베풀어 주신 양가 어머님께 감사드리고 마지막으로 사랑하는 남편에게 사랑과 고마움의 마음을 담아 전합니다.

모든 분께 진심으로 감사드립니다.

2007년 6월

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Abstract

Effects of Doxycycline and Corticotomy on Interleukin-1β Expression during Orthodontic Tooth Movement

**Backgrounds:** Cytokines are thought to play an important role in bone remodeling during tooth movement. The aim of this study was to investigate the effect of systemic administration of doxycycline and corticotomy on expression of Interleukin-1β (IL-1β) during orthodontic tooth movement.

**Methods:** Real time reverse transcription polymerase chain reaction was performed to measure messenger RNA (mRNA) expression of IL-1β at day 7 and 14 following application of orthodontic force to the maxillary first molar in 36 nine-week-old male Wistar rats. Doxycycline was administrated throughout the study in 9 animals (doxycycline + appliance group:DA) while other 9 animals served as treatment controls (no doxycycline + appliance group:NDA). In 9 of these animals corticotomy was performed after insertion of orthodontic appliances (corticotomy + appliance group:CA) while the remaining 9 animals received only the orthodontic appliance (appliance group:A). The animals were euthanized day 7 and 14 after appliance insertion and gingival tissue samples were obtained for analysis.
Results: A similar expression of IL-1β was shown at day 7 following application of force in both DA and NDA groups. IL-1β expression at day 14 was significantly decreased compared to that at day 7 for both DA and NDA groups. The increased expression of IL-1β was detected on CA group seven days after the application of force and these differences were statistically significant between these CA and A groups fourteen days after experiment.

Conclusion: The results suggest that expression of IL-1β during orthodontic tooth movement were not affected by administration of doxycycline, but the levels of cytokine both day 7 and day 14 were increased in corticotomy groups. It may support the hypothesis that osteoclast recruitment stimulated by orthodontic force was not influenced by doxycycline, but regional acceleratory phenomenon after performance of corticotomy increases bone remodeling during orthodontic tooth movement.

KEY WORDS: Doxycycline, Corticotomy, Tooth movement, IL-1β Expression
Effects of Doxycycline and corticotomy on Interleukin-1β Expression during Orthodontic Tooth Movement

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I. Introduction

Pressure onto the periodontal ligament (PDL) space induced by orthodontic forces results in vascular compression, which leads to cellular activation and the release of pro-inflammatory molecules such as prostaglandins and cytokines.¹ Prostaglandin E₂ (PGE₂) and interleukin-1β (IL-1β) levels in human gingival crevicular fluid (GCF) has been shown to be elevated during orthodontic tooth movement.² Thus it appears that these inflammatory mediators may regulate biological processes related to alveolar bone remodeling for initiation of tooth movement, although the mechanism is not yet fully understood.¹³

PGE has been known to be closely involved in bone resorption.⁴ Yamasake et al. has shown that the rate of tooth movement was almost double in sites receiving PGE₁ injections compared to vehicle-control.⁵ Conversely, anti-inflammatory drugs may reduce the rate of bone resorption by interfering with biologic mechanisms induced by
IL-1β, a major physiologic form of IL-1, is a known potent cytokine involved in initiation of bone resorption. Interestingly, IL-1β may induce elevation of PGE synthesis in a dose- and time-related order. Inversely, administration of IL-1β and/or PGE significantly enhanced the rate of tooth movement. These findings suggest that elevation of these mediator levels may serve as a marker for increased local bone remodeling.

It has been shown that systemic and local administration of pharmaceutical agents may affect tooth movement. As the use of antibiotics increases, this may cause variations in normal bone turnover in orthodontic patients. Doxycycline modulates alveolar bone metabolism, most likely due to an inhibitory effect on bone resorption including inhibition of several matrix metalloproteinases. In still other studies, doxycycline has been shown to affect osteoclast function. Tetracyclines have also been shown to reduce expression of cytokines involved in bone resorption including IL-1 and TNF-α. Minocycline added to gingival fibroblast cultures inhibited synthesis of PGE_2_. Moreover, it has been shown that minocycline may prevent a decrease of bone mineral density in ovariectomized rats.

It has been shown that healing activity was dramatically increased adjacent to the injury site in osseous tissue. This biologic process, regional acceleratory phenomenon (RAP), potentiates healing by localized burst of hard and soft tissue remodeling and involves increase in bone turnover and decrease in regional bone density. The characteristics of RAP during initial phase appears an enhanced porosity in cortical bone due to increase in osteoclastic activity. Interestingly, tooth movement
was enhanced in rats with osteoporosis. These findings suggest that localized transient osteoporosis, which means increased mobilization of calcium, decreased bone density and increased bone turnover, result in rapid tooth movement after RAP invoked by corticotomy. In still other studies, there has been strong indirect evidence that the physiologic events associated with RAP result in corticotomy-facilitated tooth movement. PGE\textsubscript{2} has also been shown to facilitate RAP in the repair of experimental fractures. However, an assessment of PGE2 and IL-1β after corticotomy have not been made to see the RAP.

Administration of doxycycline and corticotomy during orthodontic tooth movement might add interacting factors for the study of osteoclast. The aim of this study was to investigate whether systemic administration of doxycycline and corticotomy induced different gene transcription of IL-1β in the gingival tissues involved in orthodontic tooth movement.
II. Material and Methods

A. Animals

Thirty six nine-week-old, male Wistar rats, approximate weight of 280 g, from Yonsei University vivarium were used. All animals were kept in stainless-steel cages in air-conditioning and subject to standard 12-hour light/dark cycle. They were fed with a pellet diet (8811M0001, Extrusion, Superfeed Co., LTD, Gangwon, Korea) and tap water ad libitum. They were checked everyday in regard to their health status.

Doxycycline was administrated throughout the study in 9 animals (doxycycline + appliance group:DA) while other 9 animals served as treatment controls (no doxycycline + appliance group:NDA). In 9 of these animals corticotomy was performed after insertion of orthodontic appliances (corticotomy + appliance group:CA) while the remaining 9 animals received only the orthodontic appliance (appliance group:A). From each group, the animals were euthanized day 7 and day 14.

B. Experimental Procedures

A constant force of 20 g was generated using a closed coil spring (Sentalloy, 0.009 x 0.036, Tomy Incorporated, Tokyo, Japan) to move the maxillary first molar mesially. The springs were attached to the maxillary left first molar and the maxillary incisors using a stainless steel ligature wires. Light-curing bonding material (3M Unitek, Nonrovia, CA, USA) was applied on perforations produced by diamond bur along the mesio-lingual and disto-lingual line angle of the maxillary first molar and the distal sides of the incisors to ensure maximum retention of the coil spring.
(Figure 1A). Reactivation was not done during the study period. Animals scheduled for antibiotic treatment received 5 mg/kg bodyweight/day doxycycline (Dentistar, Hana Pharmaceutical company, Hwa Sung, Kyonggido, Korea) added to tap water. For CA group, the vertical corticotomy cuts without flap reflection were performed through the cortical layer of the bone using fine fissure bur (Figure 1B). A GluStitch (Salvin Dental Specialties, Inc., NC, USA) was applied to improve tissue adhesion around the wound area on the rats receiving corticotomy.

The weight of the animals was recorded on the day of the appliance insertion and before death. All procedures were performed using general anesthesia induced by subcutaneous injection of Zoletil (Tiletamine 125 ml, Zolazepam 125 ml; 0.04 ml Virbac, 060516 carros, France) and Rompun (Xylazire hydroxychloride 23.32 mg/ml; 0.01 ml Bayer AG, 51368 Leverkusen, German). At the end of each experimental period the animals were euthanized using an overdose of an anesthetic. The gingiva around the maxillary first molar was excised, frozen in liquid nitrogen and kept at -80°C until use. Real time reverse transcription polymerase chain reaction (real time RT-PCR) was used to quantify IL-1β as reported elsewhere.27,28

C. Real Time RT-PCR

1. RNA extraction

TRI zol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) was used to extract total RNA from each sample

2. cDNA synthesis

One µg total RNA was reverse transcribed into cDNA using a Promega’s
Figure 1. Schematic drawing of the rat model (A) orthodontic appliance placed (B) orthodontic appliance placed after corticotomy
Reverse Transcription system (Promega Corp., Madison, WI, USA). Reverse transcriptase was added in the mixture containing RT POX buffer, 2.5 mM MgCl$_2$, Oligo (dT) primer, dNTP Mix and Rnasin. The reagents were incubated at 42°C for 15 min, and then heated to 95°C for 5 min.

3. **Real time RT-PCR**

Real time RT-PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers and probes of IL-1β and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) are shown in Table I. PCR amplifications were performed in a total volume of 25 µl, containing 50ng cDNA sample, 1X TaqMan Universal PCR Master Mix, 300 nM of each primer and 250 nM TaqMan probe following the manufacturer’s protocol. Initial thermal cycling conditions were 2 min at 50°C and then 10 min at 95°C. Cycle conditions for IL-1β and GAPDH were 15 sec at 95°C and 1 min at 60°C, and RNAs for IL-1β and GAPDH were amplified by 40 cycles. For PCR, oligonucleotide hybridization probe was labeled with 6-carboxyfluorescein as reporter fluorescence and 6-carbocyt-tetramethylrhodamine as quencher fluorescence.

4. **Quantification using the Standard Curve Method**

For quantification of results, cDNA fragments of the known sequences were constructed as standards. The amount of GAPDH mRNA and mRNA levels of cytokine were measured using this Standard Curve method. The results were adjusted by the amount of GAPDH mRNA. Levels of these mRNA in each sample were expressed as the ratio versus that of control. Every sample was measured in triplicate.
Table 1. Genes analyzed by quantitative TaqMan PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Forward primer</td>
<td>CAA CCA ACA AGT GAT ATT CTC CAT G</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>GAT CCA CAC TCT CCA GCT GCA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CTG TGT AAT GAA AGA CGG CAC ACC CAC C</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward primer</td>
<td>CAT CTT CTC AAA ATT CGA GTG ACA A</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>TGG GAG TAG ACA AGG TAC AAC CC</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CAC GTC GTA GCA AAC CAC CAA GTG GA</td>
</tr>
</tbody>
</table>

Sequences for the primers and probes are shown in 5’ to 3’ orientation; probes contain a FAM on the 5’ end and a TAMRA on the 3’ end.

**D. Tooth Movement**

Each animal was placed in a specially made craniostat for standardized radiography. A wooden wedge was placed into each ear to ensure position. An intraoral radiographic apparatus (IMS 2000 Inermecs Co., LTD, Seoul, Korea) was used with dental film (Insight Kodak Co., Rochester, NY, USA). The amount of tooth movement was measured on lateral cranial radiographic images by one masked examiner. Horizontal and vertical reference lines were used for cephalometric measurement. The horizontal reference plane was defined as the most anterior point of the nasal bone and the most posterior point of the squama occipitalis, and the vertical reference plane was as the most superior point of the parietal bone and the most inferior point of the tympanic.
bone. The two parallel lines to the parietal-tympanic plane were used to measure the amount of mesial movement of the maxillary first molar (Figure 2). One line was drawn on the most anterior point of the anterior border of the maxillary second molar crown, and the other on the most posterior point of the posterior border of the maxillary first molar crown. The distance between the maxillary first and second molar digitized with a film scanner (PowerLook 1100, UMAX, Hyo Sung Electric Light LTD, Seoul, Korea) was measured using cephalometric image analysis (V-Ceph. Cybermed Inc, Seoul, Korea). The distance was measured thrice to statistically evaluate the reproducibility of the examiner. The examiner exhibited high reproducibility.

Figure 2. Landmarks on lateral cephalometric radiographs. Oc, most posterior point of squama occipitalis; Pa, most superior point of parietal bone; T, most inferior point of tympanic bone; Na, most anterior point of nasal bone.
E. Statistical Analysis

Data were expressed as means ± standard error of the mean and analyzed by nonparametric standard techniques such as Wilcoxon and Mann-Whitney tests as appropriate. A p-value <0.05 was required for statistical significant.
III. Results

The orthodontically treated animals drank and ate less during the first 3-4 days of study but rebounded to normal food and water ingestion and did not seem to be bothered by the orthodontic appliance, hence body weight increased over the experimental period. Four animals were excluded from the analysis due to appliance displacement.

In the NDA group, a statistically significant difference in mRNA levels for IL-1β was found between the pressure side (left side) and the contralateral control side (right side). mRNA expression of IL-1β on the pressure side was greater at 7 days compared with at 14 days, while that on the control side showed no difference between at 7 and 14 days (Figure 3). The similar results were observed in the DA group (Figure 4).

Comparing the NDA group to the DA group, no difference was found in IL-1β mRNA expression on the pressure side day 7 and 14. On day 14, IL-1β mRNA expression was statistically significantly decreased on both groups (Figure 5). On control side, there was no difference between the groups as well. On the other hand, IL-1β mRNA expression on control side of DA group revealed statistically significant increase from day 7 to day 14 (Figure 6).
Figure 3. IL-1β mRNA expression (Means and SEM) in the no doxycycline + appliance group (NDA). * P < 0.05
Figure 4. IL-1β mRNA expression (Means and SEM) in the doxycycline + appliance group (DA).  * P < 0.05
Figure 5. IL-1β mRNA expression (Means and SEM) on pressure side for the DA and NDA groups. * P < 0.05
Figure 6. IL-1β mRNA expression (Means and SEM) on control side for the DA and NDA groups. * P < 0.05
In the CA group, a statistically significant difference in IL-1β mRNA expression was found between the pressure side and the control side at day 7 and 14. IL-1β mRNA expression on the pressure side was greater at day 7 compared to day 14 (Figure 7).

Comparing the A group to the CA group, no significant difference was found in IL-1β mRNA expression on the pressure side day 7. On the other hand, IL-1β mRNA expression in the CA group showed a statistically significant expression on day 14 (Figure 8). On the other hand, IL-1β mRNA expression on control side of CA group revealed statistically significant difference on day 7 compared to that in the A group (Figure 9).

No significant difference in tooth movement was found between the DA and NDA groups day 7 and day 14 (Figure 10). However, a statistically significant increase in tooth movement was found between the A and CA groups (Figure 11).
Figure 7. mRNA levels (relative to control) for IL-1β in the corticotomy + appliance group (CA). * P < 0.05
Figure 8. mRNA levels (relative to control) for IL-1β per group on pressure side in CA.

* P <0.05
Figure 9. mRNA levels (relative to control) for IL-1β per group on control side in CA.

* P <0.05
Figure 10. Tooth movement in the DA and NDA groups.
Figure 11. Tooth movement in the CA and A group. * P <0.05
IV. Discussion

Tooth movement has, as would be expected, an impact on the magnitude of periodontal tissue response.\textsuperscript{1} This influence can be modulated by the administration of doxycycline, probably because of the differences in alveolar bone response.\textsuperscript{19,31-32} Tetracycline has been known to act as a collagenase inhibitor, and influence the recruitment and function of osteoclasts.\textsuperscript{33-43} Additionally, the absolute volume of the alveolar bone was significantly increased in rats received doxycycline under orthodontic force.\textsuperscript{19} These results elicited evaluation of the biologic process on cellular levels related to tooth movement after administration of doxycycline.

There was a statistically significant decrease in IL-1\textbeta mRNA expression from day 7 to day 14 for both DA and NDA groups. It coincides with the study in which the maximum levels in IL-1\textbeta and IL-6 were detected on day 3 following application of orthodontic force and then decreased on day 7 and 10 for return to the baseline levels.\textsuperscript{44}

There was no difference of IL-1\textbeta mRNA expression between the NDA and the DA groups at any time point. It was demonstrated that odontoclast and osteoclast recruitment stimulated by orthodontic force was not affected by doxycycline treatment on day 7.\textsuperscript{19} Therefore, it can be speculated that doxycycline treatment may not affect a biologic process related to tooth movement for short time period. However, care should be taken on administration of doxycycline for long time period because IL-1\textbeta mRNA expression on control sides in the DA group was statistically significant increased from day 7 to day 14.
IL-1β mRNA expression on pressure side at day 14 is significantly greater in the CA group compared to the A group. This trend could be explained as a reflection of the regional acceleratory phenomenon (RAP) where healing activity was dramatically increased adjacent to the injury site after surgical wounding of the osseous tissue.\textsuperscript{21,22} It was reported that evidence of RAP was first observed 10 days after mucoperiosteal surgery in rats.\textsuperscript{45} It may be interpreted that IL-1β mRNA expression in the CA group may be affected on day 14 in this study.

IL-1β mRNA expression on control side at day 7 is significantly greater in the CA group compared to the A group. A plausible explanation may be found in a previous study that tissue reactions to mechanical force were not localized to the loaded tooth.\textsuperscript{46} Moreover, it appeared that tissue responses extended to the adjacent tooth, like the whole hemimaxilla reacted to the orthodontic force applied to the first molar.

The primary aim of this study was to investigate the cytokines expression using RT-PCR and the distance between the maxillary first and second molars was measured to use as an indicator of the possible tooth movement. No statistically significant difference in the rate of orthodontic tooth movement was found between DA and NDA groups throughout the experiment. However, a statistically significant difference in the rate of orthodontic tooth movement was found between CA and A groups on day 7, although these tooth movement may not reflect the minor displacement due to inaccuracy of the assessment method.

As antibiotics including tetracycline and erythromycin are frequently used for acne
treatment, administration of antibiotics may result in variations in normal bone turnover during orthodontic treatment.\textsuperscript{15,47} Doxycycline was selected among tetracyclines for this study, in part, because of the potent collagenase inhibitor, and because it has been also known to decrease the total number of osteoclasts and prevent alveolar bone loss following periodontal surgery in rats.\textsuperscript{19} The outcome of this study could be clinically applied in that it is possible to treat patients undergoing short-term antibiotics therapy because our data seem to indicate that the levels of cytokines, after orthodontic tooth movement, were not affected during experimental periods. The rate of orthodontic tooth movement was not affected by administration of doxycycline, either. A previous study also showed that tetracycline has a beneficial effect on tooth movement by reducing the amount of root resorption.\textsuperscript{19} A plausible explanation for decreased root resorption is primarily by the inhibition of matrix metalloproteinases.\textsuperscript{14} In addition, tetracycline could prevent gingival inflammation during tooth movement.\textsuperscript{34} Taken together, the orthodontic treatment may be continued as usual for the short-term administration of doxycycline during orthodontic treatment. However, effects of doxycycline during orthodontic treatment have to be undertaken with larger number of samples before the clinical application of this concept. Future research will be also focused on the influences of cytokines on the individual activity after administration of doxycycline depending on dose and duration.

The outcome of this study could provide the biologic process for fast rate of tooth movement following application of orthodontic force combined by corticotomy. Our data seem to indicate that the levels of cytokine, after orthodontic tooth movement followed by corticotomy, were elevated for both on day 7 and on day 14. Especially, the
differences in the levels of cytokine on day 14 were statistically significant. The rate of orthodontic tooth movement was also increased in corticotomy groups. Taken together, the orthodontic treatment combined with corticotomy may be performed in cases where reduction of orthodontic therapy treatment time is required.

The cytokines levels evaluated in human GCF did not provide the information of origin in cytokines; at transcription, at translation or simply a product.\textsuperscript{3} For this reason, the mRNA expression of these cytokines using RT-PCR was needed to investigate the cellular induction of cytokines. RT-PCR has the advantage of quantification of the cytokines, especially in cases where the available samples to be analyzed are too small to quantify the cytokines at the protein level.\textsuperscript{27} In addition, RT-PCR is thought to be a very powerful and sensitive method, while the ELISA technique for cytokine protein detection allows only a limited number of cytokines.\textsuperscript{27}
V. Conclusion

Inflammatory cytokines expressed during orthodontic tooth movement can be quantified on a cellular level using RT-PCR. This study evaluated the effect of doxycycline and corticotomy on IL-1β, believed to play important roles in bone remodeling following application of orthodontic forces. The results suggest that the expression of IL-1β is not affected by doxycycline, but a statistically significant expression of IL-1β was shown at day 14 in the CA groups.

These observations support the hypothesis that osteoclast recruitment stimulated by orthodontic force is not influenced by doxycycline, and regional acceleratory phenomenon after performance of corticotomy increases bone remodeling during orthodontic tooth movement.

Moreover, our study has shown that RT-PCR appears powerful and sensitive method to quantify mRNA expression in cases where the available samples too small to quantify the cytokines at the protein level and cellular induction of cytokines with a very short half-life needs to be assessed during orthodontic tooth movement.
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2002; 35:97-104.


**Figure Legends**

Figure 1. Schematic drawing of the rat model (A) orthodontic appliance placed (B) orthodontic appliance placed after corticotomy.

Figure 2. Landmarks on lateral cephalometric radiographs. Oc, most posterior point of squama occipitalis; Pa, most superior point of parietal bone; T, most inferior point of tympanic bone; Na, most anterior point of nasal bone.

Figure 3. IL-1β mRNA expression in the no doxycycline + appliance group: NDA.
* P < 0.05

Figure 4. IL-1β mRNA expression in the doxycycline + appliance group: DA.
* P < 0.05

Figure 5. IL-1β mRNA expression on pressure side for the DA and NDA groups.
* P < 0.05

Figure 6. IL-1β mRNA expression on control side for the DA and NDA groups.
* P < 0.05

Figure 7. mRNA levels for IL-1β in the corticotomy + appliance group: CA.
* P < 0.05
Figure 8. mRNA levels for IL-1β per group on pressure side in CA. * P <0.05

Figure 9. mRNA levels for IL-1β per group on control side in CA. * P <0.05

Figure 10. Tooth movement in the DA and NDA groups.

Figure 11. Tooth movement in the CA and A group. * P <0.05
국문 요약

독서싸이클린 (Doxycycline)과 코티카토미 (Corticotomy)가 교정적 치아 이동시 Interleukin-1β(IL-1β)에 미치는 영향

연세대학교 대학원 치의학과
(지도 백형선 교수님)
임원희

싸이토카인 (cytokines)은 교정 치료시 일어나는 골 개조에 중요한 역할을 하는 것으로 알려져 있다. 이 실험의 목적은 치아 이동시 독서싸이클린 (doxycycline)의 투여와 corticotomy 시행시 Interleukin-1β(IL-1β) 표현의 변화를 보고자 한다.

9주 된 36마리의 수컷 위스타(Wistar) 쥐의 상악 제일구치에 교정력을 가한 후에 IL-1β messenger RNA(mRNA)의 표현을 보고자 real time reverse transcription polymerase chain reaction을 행하였다. 9마리의 쥐에서는 독서싸이클린 투여 없이 치아 이동을 위한 교정 장치만 걸고, 다른 9마리의 쥐에서는 치아 교정 장치와 함께 독서싸이클린을 투여하였다. 또 다른 9마리의 쥐에서는 교정 장치를 건 후, corticotomy를 시행했고, 나머지 9마리 쥐는 교정 장치만 걸었다. 6마리의 쥐는 아무 것도 시행하지 않았다. 쥐들은 교정 장치 삽입 7일 혹은 14일 후에 희생되었으며 분석을 위해 상악 제일구치 부위의 잇몸 조직을 채취하였다.
독시싸이클린을 투여한 군과 투여하지 않은 군을 비교 시 7일째에는 비슷한 IL-1β의 표현을 보였고, 14일에서는 두 그룹 모두에서 IL-1β의 양이 감소하였다. Corticotomy를 시행한 군과 시행하지 않은 군을 비교 할 때 7일과 14일에서 corticotomy를 행하고 치아 이동을 한 군에서 IL-1β의 양이 크게 증가하였다.

결론적으로, 치아 이동 시 doxycycline의 투여는 IL-1β의 표현에 영향을 주지 않았으나, corticotomy와 함께 치아 이동을 한 경우에는 IL-1β의 증가를 볼 수 있었다.

핵심되는 말: 독시싸이클린 (doxycycline), 코티카토미 (corticotomy). 치아 이동, IL-1β 표현 (expression)