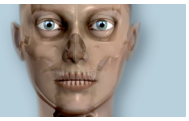
Orthodontics & Craniofacial Research



ORIGINAL ARTICLE

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Saturation of the biological response to orthodontic forces and its effect on the rate of tooth movement

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Structured Abstract

Objectives – Investigate the expression and activity of inflammatory markers in response to different magnitudes of orthodontic forces and correlate this response with other molecular and cellular events during orthodontic tooth movement.

Setting and Sample Population – CTOR Laboratory; 245 Sprague Dawley male rats.

Methods and Materials – Control, sham, and 5 different experimental groups received different magnitudes of force on the right maxillary first molar using a coil spring. In the sham group, the spring was not activated. Control group did not receive any appliance. At days 1, 3, 7, 14, and 28, the maxillae were collected for RNA and protein analysis, immunohistochemistry, and micro-CT.

Results – There was a linear relation between the force and the level of cytokine expression at lower magnitudes of force. Higher magnitudes of force did not increase the expression of cytokines. Activity of CCL2, CCL5, IL-1, TNF-α, RANKL, and number of osteoclasts reached a saturation point in response to higher magnitudes of force, with unchanged rate of tooth movement.

Conclusion – After a certain magnitude of force, there is a saturation in the biological response, and higher forces do not increase inflammatory

markers, osteoclasts, nor the amount of tooth movement. Therefore, higher forces to accelerate the rate of tooth movement are not justified.

Key words: cytokines; force; gene expression; orthodontics; osteoclasts tooth movement

Introduction

Tooth movement occurs in response to orthodontic forces. However, this movement is not completely regulated by the laws of physics and therefore is not immediate or linear in response to the magnitude of the force. The biological response plays a central role in controlling orthodontic tooth movement—the rate of bone resorption in the direction of movement determines the rate of tooth movement. Bone resorption, in turn, is controlled by the rate of osteoclast formation. Events that lead to osteoclast formation at the early stages of tooth movement emphasize the importance of inflammatory cytokines and chemokines (1, 2) in this process. In response to orthodontic forces, in non-hyalinized areas of the PDL, there is a temporary vasodilatation and release of chemokines, which recruit inflammatory cells and osteoclast precursors into the area (1). These release more inflammatory markers that directly or indirectly —through mediators such as prostaglandins— activate RANK–RANKL pathway, stimulating osteoclast precursor cells to differentiate into osteoclasts (3). The importance of cytokines can be appreciated in experiments in which inhibition of inflammatory markers blocks orthodontic tooth movement (4, 5).

If cytokines are the main signals controlling the rate of osteoclast formation during orthodontic tooth movement, the magnitude of cytokine release plays a significant role in rate of tooth movement. Increasing the cytokine release by applying small perforations to the alveolar bone (micro-osteoperforations) can significantly increase the number of osteoclasts and rate of tooth movement in both animals and humans (6, 7). However, it is not clear whether a similar phenomenon can be observed simply by increasing the magnitude of orthodontic forces. If the rate of tooth movement depends on the magnitude of the force, application of higher forces to increase the rate of tooth movement would be justified. But if this assumption is not true, then application of higher forces does not have any clinical advantage and only exposes patients to higher risk of side effects such as prolonged hyalinization and root resorption (8).

This study examined the relation between the magnitude of force and the expression of different inflammatory markers and other microscopic and macroscopic changes during orthodontic tooth movement.

Materials and methods

Animal Study

Sprague Dawley rats (245 adult males: average weight of 400 g, 120 days of age) were divided into control, sham, and different experimental groups (protocol approved by New York University Institutional Animal Care and Use Committee). Rats in the experimental groups received different magnitude of force on the maxillary right first molar (3, 10, 25, 50, or 100 cN), sham group animals received a passive spring (0 cN), and control animals did not receive any treatment. Sentalloy closing coils (GAC International, Bohemia, NY) were designed so that 1 mm activation provided required force. All coil springs were calibrated at 37 degrees with digital force gauge (Phase II Plus, Upper Saddle River, NJ, USA) to ensure consistency and reproducibility of forces. Springs were checked daily without reactivation during the experimental period using inhalation anesthesia (isoflurane). Animals with loose springs were excluded. After force application, specimens were collected at days 1, 3, 7, 14, and 28 (five animals per group). Procedures were performed on one side of the max-

illa, allowing the contralateral side to be used as internal control.

Micro-CT imaging

Maxillae were scanned with a Scanco MicroCT (μCT40; Scanco Medical, Bassersdorf, Switzerland). Results were analyzed utilizing μCT V6.0 software on the HP open platform (OpenVMS Alpha Version 1.3-1 session manager). Three reference points (buccal embrasure, middle, and palatal embrasure) were identified on the distal surface of the first molar and mesial surface of second molar at the height of contour, on occlusal sections. The average distance between those points was calculated to quantify tooth movement. The random and systematic errors were calculated using a formula described by Dahlberg and Houston (9). Both the random and systematic errors were found to be small for both intra-observer (0.013 and 0.018 mm) and inter-observer variability (0.024 and 0.022 mm).

Histology and Immunohistochemistry

Maxillae were collected at different time points and fixed in 4% paraformaldehyde, demineralized in ethylenediaminetetraacetic acid (14% EDTA) solution for 2 weeks, dehydrated in alcohol series, embedded in paraffin, and cut into 5-μm sagittal sections. Five sections were stained with hematoxylin and eosin (H&E) and scanned on a Scan Scope GL series optical microscope (Aperio, Bristol, UK) at 20× magnification. The area around the mesiopalatal root of the maxillary right first molar was divided into mesial and distal halves. The percentage of cell-free (hyalinized) area per total mesial ligament area was measured in every other section for a total of five sections. Intermediate sections were immunostained with antibodies for Cathepsin K (Millipore, Billerica, MA, USA) using Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). As negative control, sections were exposed to preimmune serum. Osteoclasts were defined as Cathepsin K–positive multinuclear cells on periosteal or endosteal bone surfaces along the full length of the mesial half of the mesiopalatal root in five sections, and values averaged for each rat. Data were expressed as the mean number of Cathepsin K–positive cells per 1 mm^2 area of periodontal ligament (PDL) and adjacent alveolar bone. Two examiners completed all histological quantifications.

RNA Analysis

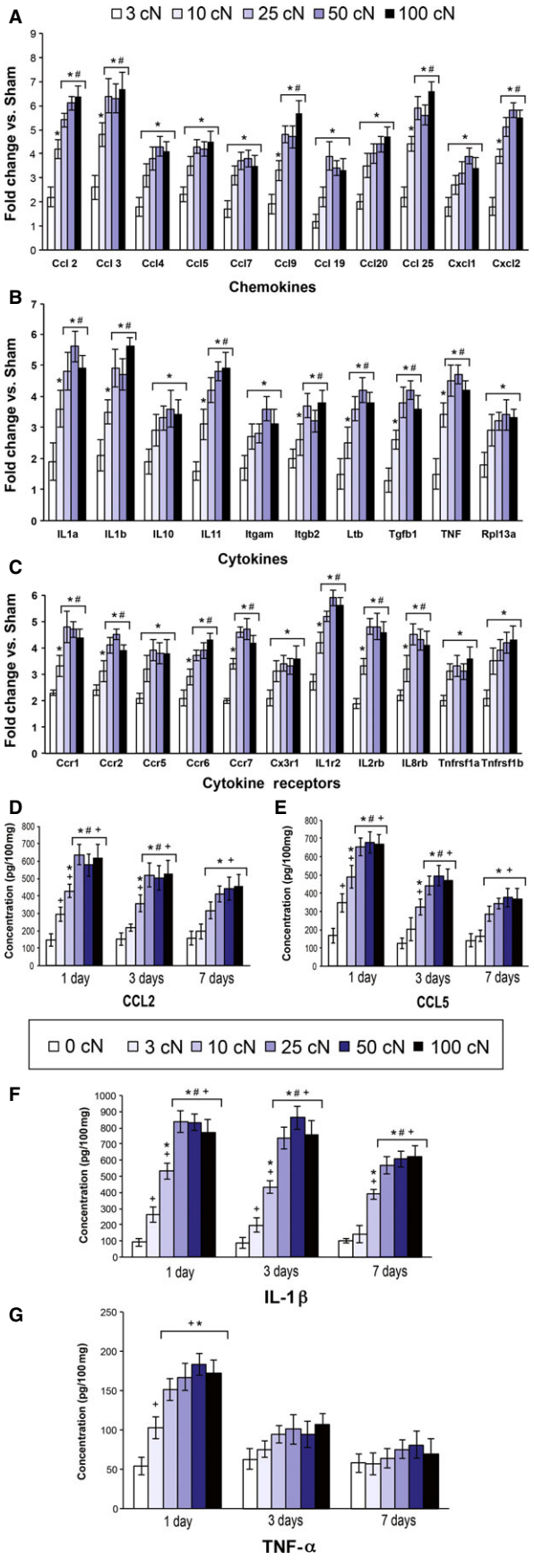
For RNA extraction, five animals from each group were sacrificed by CO_2 narcosis at 24 h, and the hemimaxillae were dissected and frozen in liquid nitrogen. Isolation of total RNA was performed as described previously (10). Eighty-six inflammatory cytokines and cytokine receptor genes were analyzed with primers specific for rat genes, with a QuantiTect SYBR Green RT-PCR kit (both Qiagen, Valencia, CA, USA) on a DNA Engine Optican 2 System (MJ Research, Waltham, MA, USA). An mRNA pool for each group was tested three times. Relative levels of mRNA were calculated and normalized to the level of GAPDH and acidic ribosomal protein mRNA.

Protein Analysis

Activity of inflammatory markers was measured by enzyme-linked immunosorbent assay (ELISA). Five hemimaxillae from each group were dissected, frozen and had tissues pulverized, lysates prepared, and total protein quantitated using a BCA protein assay kit (Pierce, Rockford, IL, USA). Concentration of interleukin (IL)-1 (Thermo, Rockford, IL, USA), tumor necrosis factor alpha (TNF-α) (Thermo), CCL5 (Abnova, Walnut, CA, USA), CCL2 (Abcam, Cambridge, MA, USA), and RANKL (MyBioSource, San Diego, CA, USA) were determined using ELISA. Data were analyzed in comparison with standard curves specific to each inflammatory marker.

Statistical Analysis

Significant differences between test groups and controls were assessed by analysis of variance (ANOVA). Pairwise multiple comparison analysis was performed with Tukey's *post hoc* test. Two-tailed p values were calculated; $p < 0.05$ was set as the level of statistical significance.



Results

Increase in magnitude of orthodontic forces does not cause linear increase in cytokines expression

Expression of 86 different cytokines, chemokines, and their receptors was evaluated 24 h after application of different force levels. In comparison with sham group, the expression of 32 chemokines (Fig. 1A), cytokines (Fig. 1B), and their receptors (Fig. 1C) increased more than twofold in experimental animals. The range of expression was 1.3- to 2.7-folds in 3 cN group, 2.2- to 4.8-folds in 10 cN group, 2.8- to 6.4-folds in 25 cN group, 3.1- to 6.3- fold in 50 cN group, and 3.1- to 6.7-fold in 100 cN group. The difference in the expression was significant between 3 cN and the other groups ($p < 0.05$), but not between 25, 50, and 100 cN for all 32 genes ($p > 0.05$). Expression of 19 genes in the 10 cN group was statistically different in comparison with those that received higher forces (25, 50, 100 cN). These results show an initial increase in the expression of inflammatory cytokines when forces increased from 3 to 10 cN, and then a plateau from 10 to 100 cN force levels.

To study the effect of magnitude of force on inflammatory markers in a longer time period, protein levels of selected chemokines and cytokines were measured by ELISA at 1, 3, and 7 days. The activity of CCL2 (Fig. 1D), CCL5 (Fig. 1E), IL-1 (Fig. 1F), and TNF-α (Fig. 1G) increased significantly for all force levels when compared to control at day 1 ($p < 0.05$). The concentration of CCL2 and CCL5 was significantly higher in 10, 25, 50, and 100 cN groups, at days 3 and 7 ($p < 0.05$).

Fig. 1. Cytokines and chemokines demonstrate saturation in expression and activity in response to higher magnitude of force. Mean 'fold' increase in expression of different chemokines (A), cytokines (B), and their receptors (C) in force groups was compared with sham group. Data are expressed as the mean ± SEM. (*, significantly different from 3 cN group; #, significantly different from 10 cN.) Mean concentration of CCL2 (D), CCL5 (E), IL-1 (F), and TNF-α (G) in the right maxillary alveolar bone after 1, 3, and 7 days of application of different magnitude of force was evaluated by ELISA. Data expressed as the mean ± SEM of concentration in picograms per 100 mg of tissue. (+, significantly different from 0 cN at same time point; *, significantly different from 3 cN at same time point; #, significantly different from 10 cN at same time point).

IL-1 concentration decreased on days 3 and 7 for all groups but was still significantly higher than sham group ($p < 0.05$) except for the 3 cN group at day 7. No differences in CCL2, CCL5, and IL-1 were observed between 25, 50, and 100 cN groups at any time point ($p > 0.05$).

TNF-α concentration showed no difference from 10 to 100 cN of force at day 1 ($p > 0.05$). At days 3 and 7 for all groups, the concentration of TNF-α significantly decreased and no statistical differences were observed between sham and experimental groups at those time points ($p > 0.05$).

Low and high magnitude of forces produced similar histological changes

We studied the cellular reaction 3, 7, and 14 days after application of different magnitude of forces. We evaluated the mesial half of the mesiopalatal root of the first maxillary molar in histological sections. At day 3 (Fig. 2A), all animals that received force showed constriction of PDL in the area adjacent to the alveolar crest. Narrowing of the PDL space was particularly obvious in experimental group that received 25, 50, and 100 cN force.

All animals presented some cell-free zones (hyalinization). The extent of this area (from the crest of the alveolar bone to the apex in the mesial side of mesiopalatal root) varied from 3, 12, 21, 23, to 26% in the experimental groups that received 3, 10, 25, 50, and 100 cN force, respectively. All increases were statistically significant in comparison with sham group ($p < 0.05$) except the 3 cN group ($p > 0.05$). The higher forces showed a significant difference in the extension of hyalinization in comparison with 3 and 10 cN ($p < 0.05$); no significant differences were observed among 25, 50, and 100 cN ($p > 0.05$).

Seven days after application of forces, all animals presented widening of PDL and areas of bone resorption from both periosteal (frontal resorption) and endosteal sides (undermining resorption) (Fig. 2C). While the areas of cell-free zone were sporadically observed, the difference between groups was not statistically significant except for the 100 cN force group that still showed a 12% increase in the cell-free zone area at day 7. At day 14, all animals showed widening of PDL due to bone resorption and no significant difference in cell-free zone area was observed ($p > 0.05$) (Fig. 2D).

Higher magnitude of force does not stimulate osteoclastogenesis markers or increase osteoclasts

To evaluate the effect of magnitude of force on osteoclastogenesis, we performed ELISA for osteoclast marker RANKL (Fig. 3A). There was no significant difference between 3 cN and sham groups at day 1 ($p < 0.05$), while the concentration of RANKL in other groups increased 3- to 3.6-fold and was statistically significant ($p > 0.05$). At day 3, the concentration of RANKL increased significantly in comparison with control ($p < 0.05$). There was no difference among the 25, 50, and 100 cN groups at days 3 and 7, but these groups showed higher concentrations of RANKL in comparison with 10 and 3 cN groups at both time points.

To investigate whether the increase in osteoclast markers was associated with increased number of osteoclasts, we conducted immunohistochemical staining for Cathepsin K. We observed an increase in the number of osteoclasts (Cathepsin K–positive cells) especially in high-stress areas—adjacent to alveolar crest in the direction of tooth movement (Fig. 3B) or in the apex area in opposite direction of tooth movement. At day 7, in the 3 and 10 cN groups many osteoclasts were located in the PDL side (frontal resorption); in the other groups, most osteoclasts were concentrated in areas adjacent to hyalinization on the endosteal side (undermining resorption). Activation of osteoclasts was proportional to the magnitude of bone resorption in the periosteal or endosteal sides.

Quantitative analysis of Cathepsin K–positive cells in the mesial PDL and adjacent alveolar bone of the mesiopalatal root of the maxillary right first molar showed an increase in osteoclast numbers in groups that received 3, 10, 25, 50, and 100 cN force, at day 7 (Fig. 3C). Numbers of osteoclasts were significantly higher in all groups in comparison with sham group ($p < 0.05$).

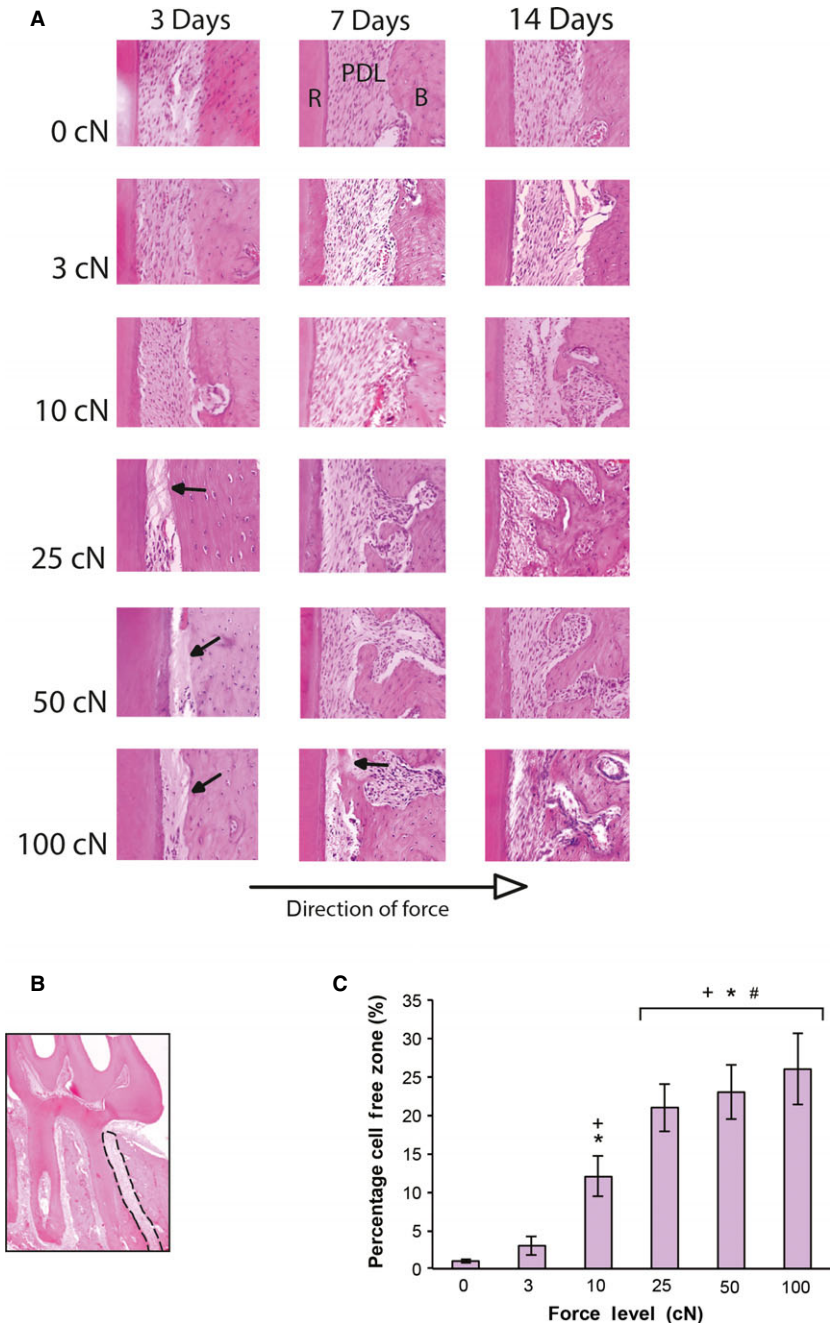


Fig. 2. Histological changes occurred in response to higher magnitude of orthodontic forces. (A) Light microphotographs of H and E stained sections at days 3, 7, and 14 after application of forces. Area shown corresponds to the mesiopalatal root of upper first molar (*R*) periodontal ligament (*PDL*) and bone (*B*). Areas of high stress close to alveolar crest show decreased PDL thickness and larger areas of cell-free zone (*black arrows* at day 3). Area of cell-free zone was quantified on mesial PDL (*black dashed line*) and data presented as percentage of cell-free zone in the total area (B). Each value represents the mean ± SEM of five animals (C). (+, significantly different from 0 cN; *, significantly different from 3 cN; #, significantly different from 10 cN.)

Different magnitude of force produces similar tooth movement

To evaluate the relevance of molecular, cellular, and histological changes in response to different orthodontic forces, we measured the magnitude of tooth movement using mCT, at days 14 and 28 (Fig. 4). All groups showed a significant increase in the distance between first and second right maxillary molars in comparison with sham ($p < 0.05$). No significant differences were observed between animals that received 10, 25, 50, and 100 cN force ($p > 0.05$) at days 14 (Fig. 4A) or 25, 50, and 100 cN force at day 28 (Fig. 4B).

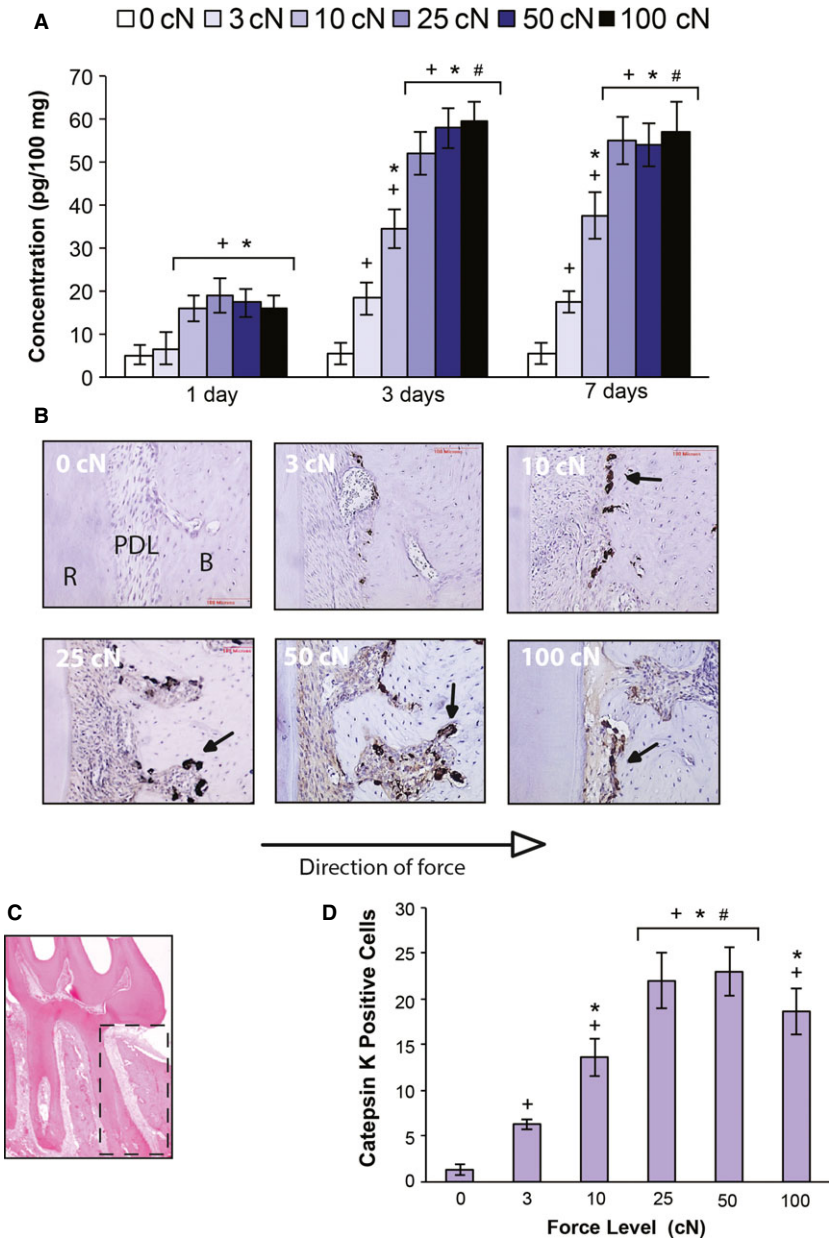


Fig. 3. Osteoclast markers and number of osteoclasts show saturation in response to higher magnitude of forces. (A) Mean concentration of RANKL in the right maxillary alveolar bone after 1, 3, and 7 days as measured by ELISA. The data are expressed as the mean ± SEM of RANKL concentration in picograms per 100 mg tissue. (+, significantly different from sham at same time point; *, significantly different from 3 cN at same time point; #, significantly different from 10 cN at same time point.) (B) Light microphotographs of Cathepsin K–positive osteoclasts in immunohistochemical stained sections of mesiopalatal root of maxillary molar. Images were collected close to the alveolar crest 7 days after application of force. Osteoclasts are stained as brown cells (*black arrows*) in sections from different force groups (0 to 100 cN). (C) Mean numbers of osteoclasts at 7 days, in PDL and adjacent alveolar bone of mesiopalatal root of maxillary molar (dashed rectangle area). (D) Each value represents the mean ± SEM of five animals (+, significantly different from 0 cN; *, significantly different from 3 cN; #, significantly different from 10 cN).

Discussion

One of the main controversies in biology of tooth movement is the relation between magnitude of force and the rate of tooth movement. Many have shown that application of higher forces does not increase the rate of tooth movement (11, 12), and others have argued the opposite (13). The use of amount of tooth movement to measure the effect of magnitude of force on the rate of tooth movement is responsible for this paradox. Although tooth movement is the desired result of the biological response to forces, it may

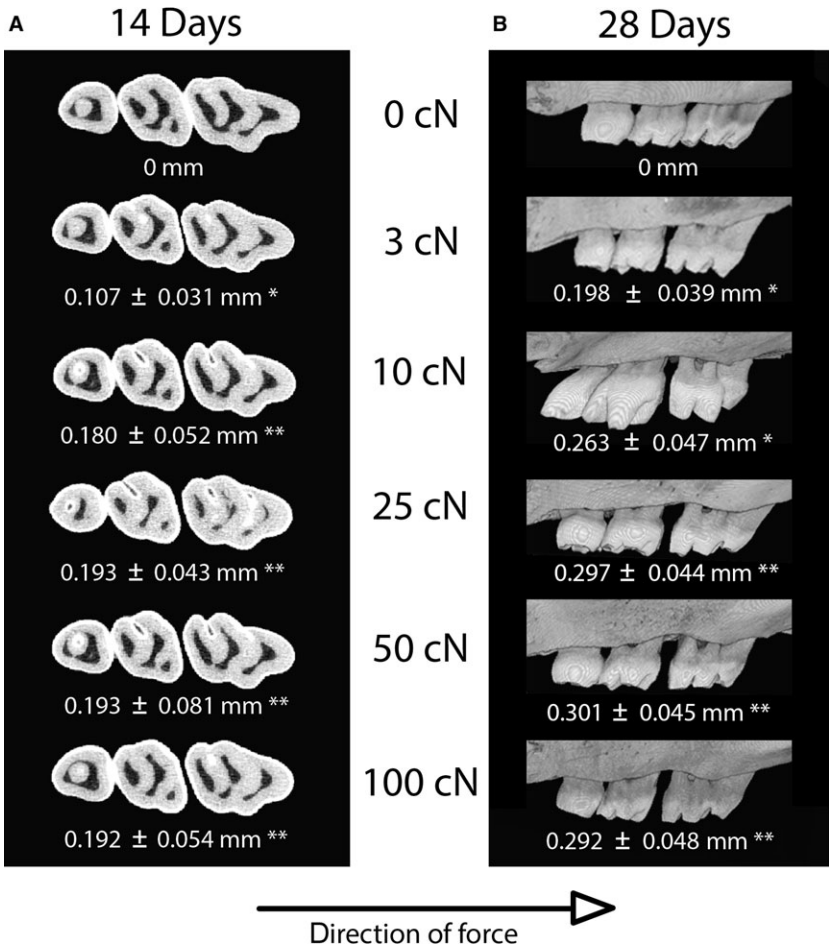


Fig. 4. Increasing the magnitude of orthodontic forces did not increase the rate of tooth movement. (A) Occlusal sections of right maxillary molars of sham and different experimental groups were obtained by micro-CT analysis 14 days after application of force. The distance between teeth was measured at height of contour from the distal surface of the first molar and mesial surface of second molar. The average distance for three measurements was calculated. (B) MicroCT 3D reconstruction of buccal view of right maxillary molars after 28 days. Each value represents the mean ± SEM of the average distance between first and second molar measured at height of contour in five animals (*, significantly different from sham; **, significantly different from sham and 3 cN).

not necessarily be a precise representative of the relation between magnitude of force and biological response that cause tooth movement. Many factors can affect the amount of tooth movement independent of the magnitude of the force. These factors can be intrinsic such as, differences in the shape of root and alveolar bone, or bone density, or extrinsic such as occlusal forces, chewing habits, or limitation of the mechanical design. These variables are more prominent in human studies where it is more difficult to obtain a large group of subjects with similar anatomical features, age, gender, and type of malocclusion. While these limitations are easier to control in animal models, depending on the duration of study, measuring tooth movement as the sole representative of the effect of magnitude of force can still produce conflicting results, because the biological response differs at different stages of tooth movement. Depending on the duration of the study, different investigators may capture different stages of this biological response and make erroneous conclusions not representative of the complete process. In our study, we investigated the biological response to different magnitude of forces in rats with similar genetic background and used molecular and cellular changes as the comparative parameters, and not just amount of tooth movement.

Our model produced uncontrolled tipping of molars as the force did not pass through the center of resistance of the tooth. Due to the dimensions of rat's maxilla and teeth, the application of other types of tooth movement was

not feasible. Uncontrolled tipping causes higher stresses in the area of alveolar crest in the direction of the applied force, and in apex area in the opposite direction of the applied force (14) with minimum stress around the tooth's center of rotation (15).

At the molecular level, 24 h after application of different magnitude of forces, the expression of inflammatory markers was stimulated, as seen in previous studies (16). At the beginning, a linear relation between magnitude of force and expression of inflammatory markers was observed, but this relation changed and inflammatory response plateaued with higher magnitude of orthodontic forces, in both short and longer time periods. This plateau occurred between 10 and 25 cN of force. While we did not investigate forces in between that range, our results establish 25 cN as an excessive force for tooth movement studies in the rat model. We looked at the overall profile of inflammatory markers in the surrounding PDL and alveolar bone of the hemimaxilla, and not the distribution pattern of these markers in different areas of the periodontium. During tooth movement, the PDL and alveolar bone are exposed to different types of stress (17). The influence of each stress type in the expression of these inflammatory markers was not addressed in this study.

The increase of inflammatory markers was accompanied with a similar increase in the activity of RANKL, which, through interaction with RANK, plays an important role in the activation of osteoclast precursor cells. Both RANKL activity and the number of osteoclasts showed saturation in response to higher magnitude of forces. The number of osteoclast was slightly lower in 100 cN force at day 7, which could be attributed to the larger area of cell-free zone that was observed. However, at day 14, the extent of the hyalinization area in all groups decreases significantly and the histological changes were very similar. As the osteoclasts control the rate of tooth movement, we expected similar number of osteoclasts to produce similar rates of tooth movement, as seen in our long-term experiments.

If application of higher forces does not increase the activity of inflammatory markers and the cascade of molecular and cellular events that follows, application of higher forces cannot increase the rate of tooth movement and can only expose the tooth to increased risk of side effects such as root resorption. Indeed, the experimental group that received 100 cN showed larger areas of root resorption in comparison with other groups (data not shown) in agreement with previous observations (18).

Conclusions

Increasing the magnitude of orthodontic force cannot increase the biological response, and therefore, it cannot be justified as a methodology to increase the rate of tooth movement. To increase the rate of tooth movement, the saturation of the biological response must be overcome by other methods.

Clinical relevance

Inflammatory markers play an important role during tooth movement by controlling the rate of osteoclast formation and therefore bone resorption. Some may assume that increasing the magnitude of orthodontic forces may increase the expression of inflammatory markers and the rate of tooth movement. We show that, in response to higher magnitude of forces, a saturation point in the biological response is reached where no further increase in inflammatory markers or tooth movement is observed. Therefore, higher forces to increase the rate of tooth movement are not justified, and other methods should be considered.

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