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

A Thesis Presented by

Michelle Yuching Chou, DDS, MPH

to

The Faculty of Medicine

In partial fulfillment of the requirements

for the degree of

Doctor of Medical Sciences

Research Mentors: Mani Alikhani, DDS, MSc, PhD
Visiting Associate Professor of Developmental Biology, Harvard School of Dental Medicine
Associate Research Investigator, The Forsyth Institute
Director, Consortium for Translational Orthodontic Research

Mohamed I. Masoud, BDS, DMSc
Lecturer of Developmental Biology
Program Director of Advanced Graduate Education in Orthodontics

Harvard School of Dental Medicine

Boston, Massachusetts

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Dedication

This dissertation is dedicated to my dear mother, Ms. Yen-Ching Cheng, and my late father, Mr. Chen-Dong Chou, for their love, care and guidance in my life. I would not have become who I am without their support and sacrifice. My mother in particular, as a single parent since I was ten years old, has strived until today to provide me with unconditional support and opportunities that she could never get from her previous generation. Either to become a concert pianist, an orchestra conductor, or an orthodontist as my career goal has evolved, she has always encouraged me to not only endeavoring for success, but being a moral and responsible person who can contribute to the society.

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Abstract

Objectives: The objectives of this research are to investigate the biological response of the body to different magnitude of force, and to investigate if the response varies among individuals. Therefore this research has 3 Specific Aims: Aim 1 is to investigate the biological response to different magnitudes of orthodontic forces at molecular and cellular levels in animals; Aim 2 is to investigate whether an equal magnitude of force can stimulate different levels of biological response among individuals; Aim 3 is to investigate whether the limit of biological response to different magnitudes of orthodontic force varies among individuals.

Methods and Materials: For Aim 1, different magnitudes (0 to 100 cN) of constant, continuous force were applied on the maxillary first molar of Sprague Dawley rats. The maxillae were collected for RNA and protein analysis, immunohistochemistry, and micro CT at different time points. For Aim 2, human subjects in different age groups (age 11-14 and 21-45) were recruited. Canine retraction was rendered with a constant force of 50 cN, and gingival crevicular fluid (GCF) was collected at different time points up to 28 days after retraction. The activity of inflammatory markers in GCF including IL-1β, CCL2, TNF-α, RANKL, and MMP-9 were measured using protein arrays. The rate of canine retraction in 28 and 56 days was measured on study models. For Aim 3, human subjects in different age groups (same as Aim 2) were recruited. Each subject in both age groups was randomly assigned to receive certain magnitude of constant force (50 to 200 cN) for canine retraction. The activity of different inflammatory markers in GCF one day after retraction was measured using protein arrays. The rate of canine retraction in 28 days was measured on study models.

Results: In the animal study, 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, RANKL, and number of osteoclasts reached a saturation point in response to higher magnitudes of force, with unchanged rate of tooth movement. In the clinical studies, activities of IL-1β, CCL2, TNF-α, RANKL, and MMP-9 increased significantly one day after retraction in both age groups. Inflammatory marker activities were significantly higher in adults compared with adolescents at 50-cN force. However, the rate of tooth movement was greater in adolescents than adults during the 56-day study period. At higher force magnitudes, the inflammatory marker activities were higher in adolescents than adults. Both age groups demonstrated saturation in biological response, with higher saturation point in adolescents than adults.

Conclusion:

1. After a certain magnitude of force, there is a saturation in the biological response, where higher magnitude of force does not increase inflammatory markers, osteoclasts, nor amount of tooth movement. Therefore, higher forces to accelerate the rate of tooth movement are not justified and other methods should be considered.
2. The level of biological response varies among individuals to an identical magnitude of force. Therefore, one should compare the level of biological response within the same individual.
3. Saturation of biological response to higher magnitude of orthodontic force exists in both rats and humans, and the saturation point varies among individuals. Adolescents exhibit higher saturation point than adults. Therefore it is not justified to apply higher magnitudes of force in adults.

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

Chapter 1. Introduction and Review of Literatures

1.1. Introduction

Orthodontic treatment is made possible by the nature of tissue adaptation and bone remodeling capabilities upon force application on teeth and bone. However, the relation between magnitude of force and consequent biologic adaptation is unclear. Understanding about such relation is essential for determining how much force should be applied to optimize the rate of orthodontic tooth movement (OTM), and in turn shorten the duration of treatment.

Duration of treatment is one of the most challenging aspects of orthodontic treatment since prolonged treatment duration leads many patients, especially adults, to either avoid treatment, or to seek alternative solutions with less than optimal results (Uribe et al. 2014). Therefore, optimizing OTM without increasing potential risk factors or compromising treatment outcomes remains one of the main challenges in orthodontic research today. To address these challenges, understanding the biological basis of OTM is essential.

If the rate of tooth movement depends on force magnitude, it follows that the application of higher forces to increase the rate of tooth movement is justified. On the other hand, if this assumption is not true, then the application of higher forces does not provide any clinical advantage and only exposes patients to an increased risk of side effects, such as root resorption (Fox 2005; Roscoe et al. 2015; Sameshima and Sinclair 2001; Segal et al. 2004; Uribe et al. 2014; Weltman et al. 2010). Current literature relating force magnitude to the rate of tooth movement has produced contradictory results (Quinn and Yoshikawa 1985; Ren et al. 2002; Yee et al. 2009). Therefore, this field warrants further research.

In addition, animal studies have shown that even with standardized, constant, and equal

forces, the rate of orthodontic tooth movement can vary substantially among and even within subjects (Pilon et al. 1996; van Leeuwen et al. 1999). Therefore, understanding the effect of individual variations on the biological response to orthodontic force will help clinicians to make optimized and customized treatment decisions for their patients, and improve the treatment efficiency by modifying the current treatment system and modalities.

Ultimately, by understanding biology of tooth movement in depth, revolutionary, customized treatment system in orthodontics can be inspired and evolved.

1.2. Biological Basis of Orthodontic Tooth Movement

It is generally accepted that for orthodontic force to move a tooth, bone resorption should be activated to remove the bone in the compressive path of movement, while bone formation should follow on the opposite tension side of the tooth to maintain the integrity of alveolar bone. It is the rate of bone resorption that controls the rate of tooth movement, while the rate of bone formation determines the success of treatment. Based on these concepts, the biological events of orthodontic tooth movement can be divided into two main phases: a catabolic phase when bone resorption occurs, and an anabolic phase when bone formation occurs.

In spite of clarity in the overall cellular and histological events of orthodontic movement, the mechanism behind these events is ambiguous. Some of the questions that remain less agreed upon include: How are bone resorption and formation activated in response to orthodontic forces? Are these events the direct effect of mechanical stimulation induced by orthodontic forces or are there indirect mediators of orthodontic tooth movement? Does the periodontal ligament (PDL) play a role in controlling the rate of tooth movement? How can the catabolic and anabolic effects

of orthodontic forces be increased when needed? To address these questions a general understanding of how each type of bone cell functions is necessary.

1.2.1. Bone cells and their role in biology of tooth movement

Bone is a dynamic tissue that remodels in response to mechanical force. The cells that perform this response are distributed throughout the bone and each is specialized to perform specific functions needed to detect force (both its magnitude and direction), recruit cells that resorb bone at specific sites, and activate cells to deposit new bone matrix and promote mineralization that will withstand mechanical force.

Three types of bone cells play a significant role in the biology of tooth movement: osteoblasts, osteocytes and osteoclasts. The bone-forming cells are osteoblasts, which spend their lives attaching to the bone surface. The mechanosensors are osteocytes, which are by far the most numerous bone cells in the body, but are also the least studied because they are embedded entirely within the bony matrix. The bone-resorbing cells are giant multinucleated osteoclasts, which are found on the bone surface at resorption sites. In addition, inflammatory cells (specifically, T lymphocytes and macrophages) that reside in the bone marrow are important regulators of osteoclasts and osteoblasts.

Osteoblasts are mononuclear cells found along the surface of bones. They are derived from mesenchymal stem cells in the bone marrow and synthesize collagenous and non-collagenous proteins that comprise the organic bone matrix, the osteoid. Inactive osteoblasts that cover bone surfaces, particularly in the adult skeleton, are called bone-lining cells. These cells are quiescent until growth factors or other anabolic stimuli induce their proliferation and differentiation into cuboidal osteoblasts. Osteoblasts are the main cells participating in the

anabolic phase of orthodontic tooth movement with a limited role during catabolic phase. Therefore, they are not the cells that control the rate of tooth movement.

Osteocytes are mature osteoblasts embedded in lacunae within the bone matrix. Although immobile, osteocytes possess exquisitely fine processes, which traverse the mineralized matrix in tunnels called canaliculi, to make contact with other osteocytes, as well as with osteoblasts residing on the bone surface. Given their preponderance in bone, and their intricate three-dimensional network, osteocytes are key mechanosensors that recognize mechanical load, and by regulating osteoclast and osteoblast activity, reshape the bone to fit the mechanical demand.

The mechanism by which mechanical stimulation activates osteocytes is not clear. Loading of bone under physiologic condition results in strain, or deformation, in the bone matrix, and the lacunae and canaliculi that surround the osteocytes. Some authors suggest that it is the magnitude of the matrix deformation (strain), rather than lacunae or canaliculi deformation, that triggers bone remodeling (Masella and Meister 2006; Mosley et al. 1997). Conversely, others argue that load itself is not the main ostoeogenic component of mechanical stimulation but, instead, load by-products such as strain rate (O'Connor et al. 1982), strain distribution (Rubin and Lanyon 1987) or fluid flow (Qin et al. 2003) are the primary remodeling initiators. While this controversy remains under active investigation, there is consensus that mechanical stimulation is detected by osteocytes via fluid shear stress produced by increased fluid flow in the lacuno-canalicular system, and electrical strain potentials. These responses to mechanical load activate osteocytes to secrete key factors, such as prostaglandins, nitric oxide or insulin-like growth factors (IGFs), which then activate osteoclasts and osteoblasts in a tightly synchronized biological phenomenon called bone remodeling. Under the influence of orthodontic forces, osteocytes play a critical role in detecting force and activating osteoclast–osteoblast coupling,

but they are not the cells that regulate the rate of tooth movement. They may play a role in the catabolic phase of movement by activating osteoclasts. However, it is more probable that they play a role in the anabolic phase by coordinating osteoblast activation.

The last cell type that plays a significant role in orthodontic tooth movement is the **osteoclast**, which is the major bone-resorbing cell. Osteoclasts are specialized monocyte/macrophage family members of hematopoietic origin, formed by the fusion of numerous monocytic precursors to create giant multinucleated cells. Terminal differentiation in this lineage is characterized by the acquisition of mature phenotypic markers, such as the calcitonin receptor, tartrate-resistant acid phosphatase (TRAP), and the appearance of a ruffled border rich in proton pumps that acidify the bone surface to which the cells are attached, resulting in resorption pits.

Osteoclasts play an important role in the catabolic phase of orthodontic tooth movement. In fact, it is osteoclasts that control the rate of bone resorption and, therefore, the rate of tooth movement. However, osteoclasts do not function independently. In fact, they require signals from other cells for their maturation, activation and targeted, site-specific bone resorption. The consequences of unregulated osteoclast activation would be catastrophic as bone resorption would proceed unchecked producing weakened bone and fractures. Consequently, osteoclasts cannot be considered the direct target of orthodontic forces. Instead, the upstream events that control osteoclast formation and activation must be the main target, but what these upstream events are remains controversial.

When viewed physiologically, normal healthy bone remodeling is a tightly choreographed sequence of cellular activity. Mechanical force distorts osteocytes housed in lacunae and canaliculi, often producing micro-fractures, which are cleared out by osteoclasts.

Osteoblasts follow to fill in the newly excavated site. Some of those osteoblasts become embedded in the new bone to form new osteocytes to replace those lost at the remodeling site. Thus, healthy strong bone that can withstand mechanical force applications is formed due to signaling between osteocytes, osteoclasts, and osteoblasts. As we will discuss below, a variation of this response, which incorporates immune cells and inflammatory cytokines, is key to understand the biology of tooth movement.

1.2.2. Catabolic phase of orthodontic tooth movement

1.2.2.1. Theories on initiation of orthodontic tooth movement

Orthodontic forces produce different types of movement depending on the magnitude of forces and couples applied to the teeth. Each type of tooth movement causes a specific pattern of stress distribution in different areas of the PDL and alveolar bone. It is widely accepted that the areas experiencing the highest compression stresses are the ones that undergo the highest levels of osteoclastic bone resorption. During recent years, many theories have been developed to explain the initial events of orthodontic tooth movement leading to osteoclast activation in these compression sites. In general, these theories split into two camps: one proposes that bone cells (more specifically osteocytes) are the direct target of orthodontic forces (**Direct View**), while the other proposes that the PDL is the key target of treatment (**Indirect View**). However, there is agreement in both theories that osteoclasts are the final cells that resorb bone, and therefore, are the cells that control the rate of tooth movement (Teixeira and Alikhani 2016).

Using the research on weight-bearing bone as the basis of the **Direct View** hypothesis, its proponents claim that there are two mechanisms by which direct loading may activate **osteocytes**. In the first mechanism, when mechanical stimulation is at physiological levels, osteocytes

recognize the different components of mechanical stimulation (such as matrix deformation) and direct the bone remodeling machinery by triggering osteoclast to remove the old bone structure and rebuild new load-friendly bone by activating osteoblasts. According to this mechanism, orthodontic tooth movement can be considered a physiologic adaptation to mechanical stimulation induced by orthodontic forces. In the second mechanism, when mechanical stimulation is at higher (pathologic) load levels, micro-fractures appear in the matrix that are recognized by osteocytes, which then activate the remodeling machinery. In this mechanism, orthodontic tooth movement is considered a response to trauma caused by orthodontic forces.

While the osteocyte-driven bone remodeling response to physiologic or pathologic levels of forces is supported by data derived from studies of weight-bearing bones, this theory of bone remodeling in response to orthodontic forces is questionable. Experiments in long bones and alveolar bone demonstrate that at physiologic levels osteocytes do not recognize static forces (Alikhani et al. 2012; Rubin and Lanyon 1984). This argues against considering orthodontic tooth movement as a physiologic adaptation to mechanical stimulation, since orthodontic forces are mostly static rather than intermittent, as long bones would experience. Supporting this idea, application of orthodontic forces to dental implants used as anchorage during orthodontic treatment, does not induce movement of the implant.

Can orthodontic forces stimulate tooth movement by inducing microfractures in bone? While microfractures occur in response to orthodontic forces (Verna et al. 2005), the possibility that it is the main mechanism of tooth movement is low. The fact that orthodontic force cannot move an ankylosed tooth demonstrates that microfractures are not the main triggers for tooth movement. If microfractures are the trigger for tooth movement, one would expect higher forces should increase the rate of movement. It should be emphasized that while application of higher

force magnitudes (at the pathologic level) may damage the bone around an implant significantly to the point of implant failure, the stronger forces do not move the implant in bone. Coupled with the fact that the lower, physiological, magnitude of force is applied during clinical orthodontics, strongly suggests that microfractures are not the trigger for orthodontic tooth movement.

Supporters of the **Indirect View** of tooth movement propose that the **PDL** is the primary target of orthodontic forces. Consider the impossibility of moving an ankylosed tooth, which lacks a PDL. Based on this proposal, the PDL exhibits areas of compression and tension in response to orthodontic forces. If the duration of force application is limited to a few seconds (i.e., is intermittent), the incompressible tissue fluid prevents quick displacement of the tooth within the PDL space. However, if the force on a tooth is maintained (i.e., is static, as in orthodontic treatment), the fluid is squeezed out of the PDL, providing space for tooth displacement in the socket and further compression of the PDL. The immediate result of this displacement is blood vessel constriction in the compression site. The resulting decreased blood flow would cause a decrease in nutrient and oxygen levels (hypoxia). Depending on the magnitude of pressure and blood flow impairment, some of the cells go through apoptosis, while other cells die non-specifically, resulting in an area of necrosis that is identified histologically as the “cell-free zone” (Fig. 1-1). It should be emphasized that apoptotic or necrotic changes are not limited to PDL cells and some of the osteoblasts and osteocytes in adjacent alveolar bone also die in response to orthodontic forces. This sequence of events leads to an aseptic, acute inflammatory response with the early release of chemokines from local cells (Fig. 1-2).

Chemokines are small proteins released by local cells that can attract other cells to the area. The release of chemokines in response to orthodontic forces facilitates expression of adhesion molecules in blood vessels and stimulates further recruitment of inflammatory and

precursor cells from the microvasculature into the extravascular space. Given their strong biological influence on localized cellular activity, it is important to discuss chemokines in the context of the biology of tooth movement.

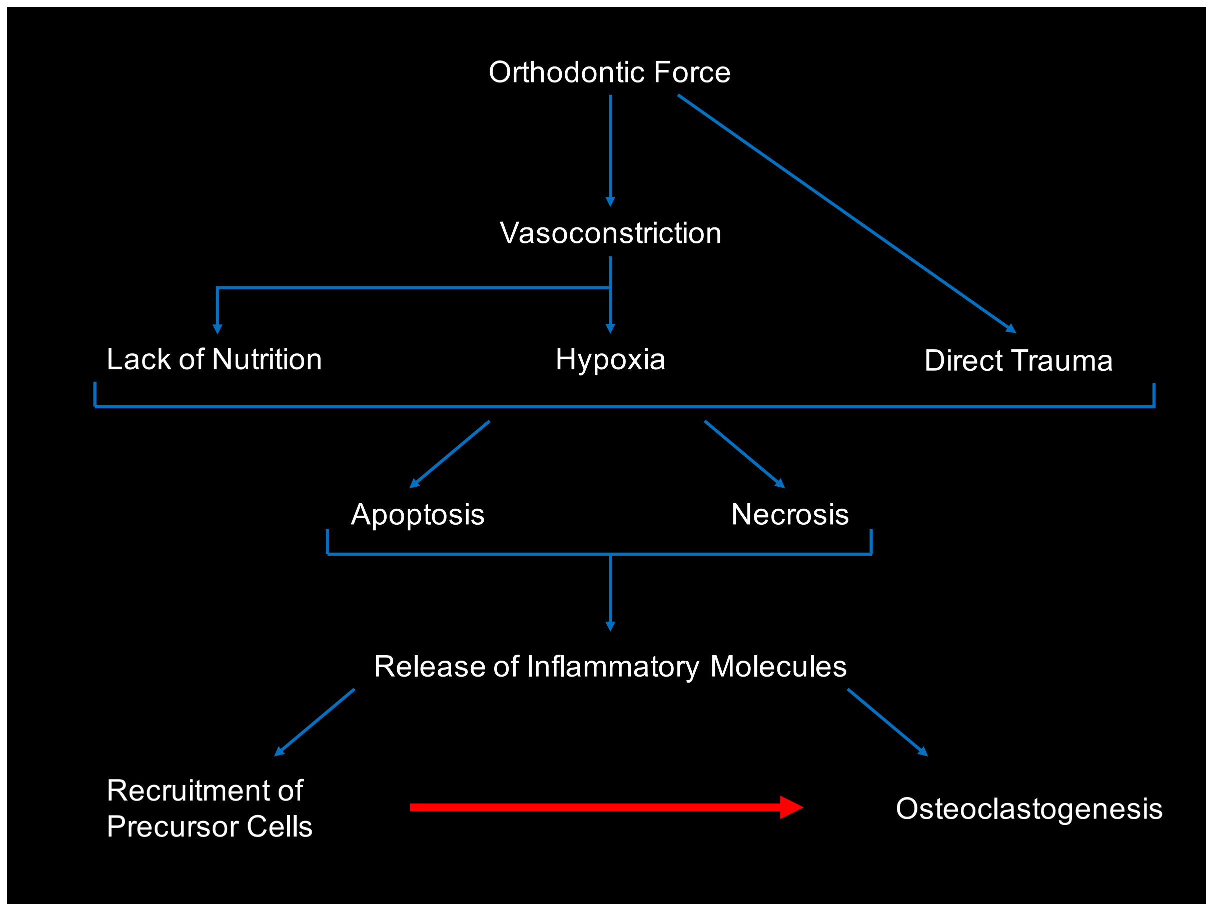


Figure 1-1. **Diagram of cellular events in the compression side in response to application of orthodontic force.**

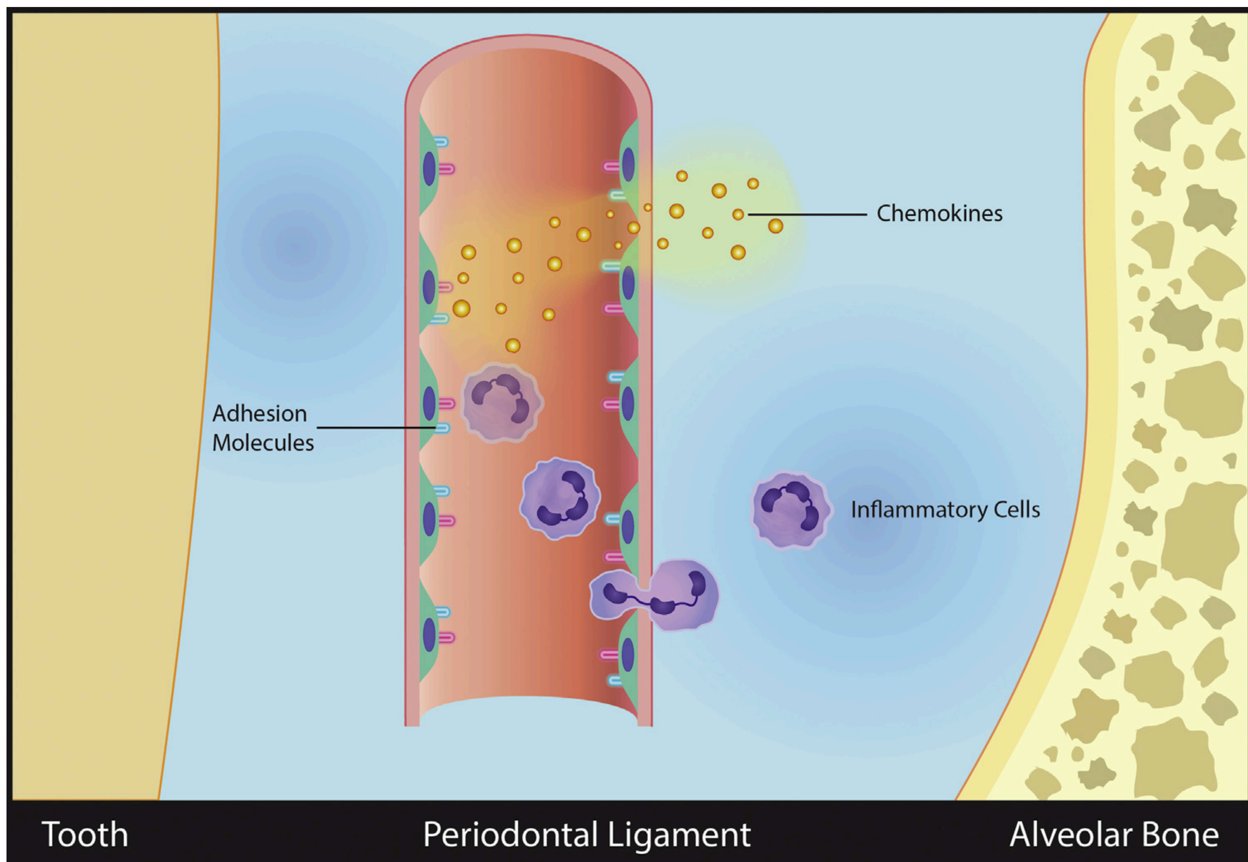


Figure 1-2. Schematic representation of increase in permeability of vessels, release of chemokines, expression of adhesion molecules, and recruitment of inflammatory and precursor cells during early events of orthodontic tooth movement (Alikhani et al. 2015b).

1.2.2.2. Initial aseptic inflammatory response to orthodontic force

One of the chemokines that is released during tooth movement is monocyte chemoattractant protein-1 (MCP-1 or CCL2) (Taddei et al. 2012), which plays an important role in recruiting monocytes from the bloodstream to enter the surrounding tissue where they become tissue macrophages or, importantly to us, osteoclasts. Similarly, the release of CCL3 (Taddei et al. 2013) and CCL5 (RANTES) (Andrade et al. 2009) during orthodontic tooth movement leads to osteoclast recruitment and activation.

Within the first few hours of orthodontic treatment there is further release of a broad spectrum of inflammatory mediators. Thus, in addition to chemokines, cytokines are also released during orthodontic treatment. These extracellular proteins play an important role in regulating the inflammatory process. Many cytokines are pro-inflammatory and help to amplify or maintain the inflammatory response and activation of bone resorption machinery. Importantly, cytokines are anti-inflammatory, thereby preventing unrestrained progression of the inflammatory response. The main pro-inflammatory cytokines that are released during orthodontic tooth movement are IL-1α, IL-1β, TNF-α and IL-6 (Garlet et al. 2007). These cytokines are produced by inflammatory cells such as macrophages, and by local cells such as osteoblasts, fibroblasts and endothelial cells.

Another series of inflammatory mediators that are released during orthodontic tooth movement are prostaglandins (PGs) and neuropeptides. PGs are derived from arachidonic acid metabolism and can mediate virtually every step of inflammation such as vasodilation, increase vascular permeability, and adhesion of inflammatory cells. During orthodontic tooth movement, these mediators can be produced directly by local cells or by inflammatory cells in response to mechanical stimulation, or indirectly by cytokines. For example, TNF-α is a potent stimulator of

PGE_2 formation (Perkins and Kniss 1997). PGs act locally at the site of generation, then decay spontaneously or are enzymatically destroyed (Dubois et al. 1998; Ricciotti and FitzGerald 2011). Similar to PGs, neuropeptides can participate in many stages of the inflammatory response to orthodontic forces. Neuropeptides are small proteins, such as substance P, that transmit pain signals, regulate vessel tone and modulate vascular permeability (Lundy and Linden 2004). The importance of all these inflammatory makers can be appreciated in the role that they play in osteoclastogenesis.

1.2.2.3. Cytokines and inflammatory mediators governing osteoclastogenesis

As previously discussed, osteoclasts are multinucleated giant cells derived from hematopoietic stem cells of the monocyte-macrophage lineage that resorb bone. After recruitment to the compression sites, osteoclast precursors begin to differentiate into osteoclasts. Cytokines are important mediators of this process. For example, TNF-α and IL-1 bind to their receptors, TNFRII (Fuller et al. 2006) and IL-1R (Jimi et al. 1996), respectively, and directly stimulate osteoclast formation from precursor cells and osteoclast activation (Fig. 1-3). Additionally, IL-1 and IL-6 (O'Brien et al. 1999) can indirectly stimulate local cells or inflammatory cells to express macrophage colony-stimulating factor (M-CSF) and RANKL (receptor activator of nuclear factor κ□□ ligand). These ligands, through cell-to-cell interactions bind to their respective receptors, c-Fms and RANK, which are both expressed on the surface of osteoclast precursors (Fig. 1-4).

Other inflammatory mediators that enhance osteoclast formation through enhancing RANKL expression by stromal cells are PGs, especially PGE_2 (Suzawa et al. 2000). As

mentioned before, PGs can be produced by local cells directly in response to orthodontic forces or indirectly as down-stream of cytokines such as TNF-α. It should be emphasized that local cells normally downregulate osteoclastogenesis by producing a RANKL decoy receptor, osteoprotegerin (OPG) (Yasuda et al. 1998). Therefore, OPG levels in compression sites should decrease to enable tooth movement.

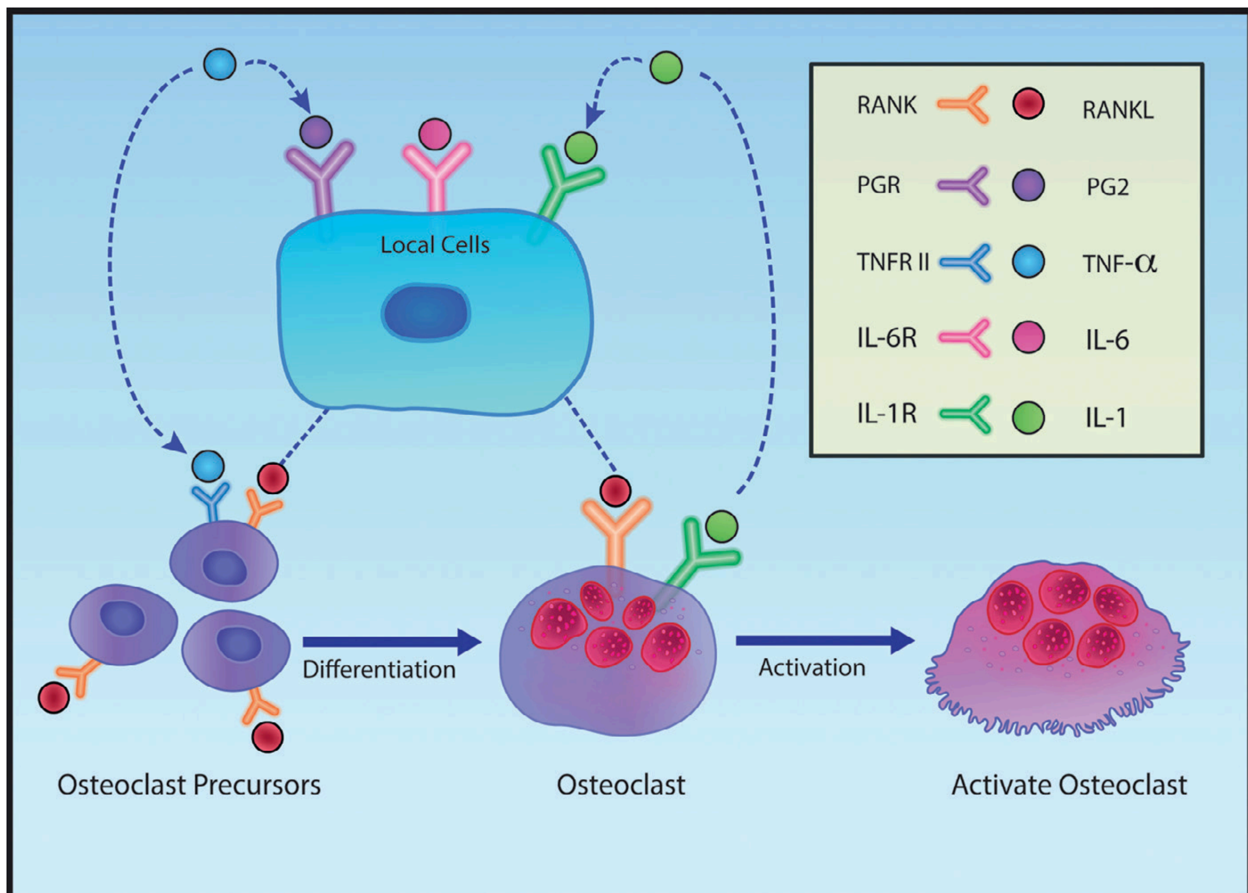


Figure 1-3. Cytokines regulate osteoclastogenesis. Cytokines are important mediators of osteoclastogenesis with important roles at different stages of this process. Some of these cytokines produced by local cells bind to receptors on the surface of osteoclast precursor cells to induce their differentiation into osteoclasts (RANKL, TNF-α), while others directly stimulate osteoclast activation (RANKL, IL-1). Additionally, local cells can also down regulate osteoclastogenesis by producing a RANKL decoy receptor, osteoprotegerin (OPG) (Alikhani et al. 2015b).

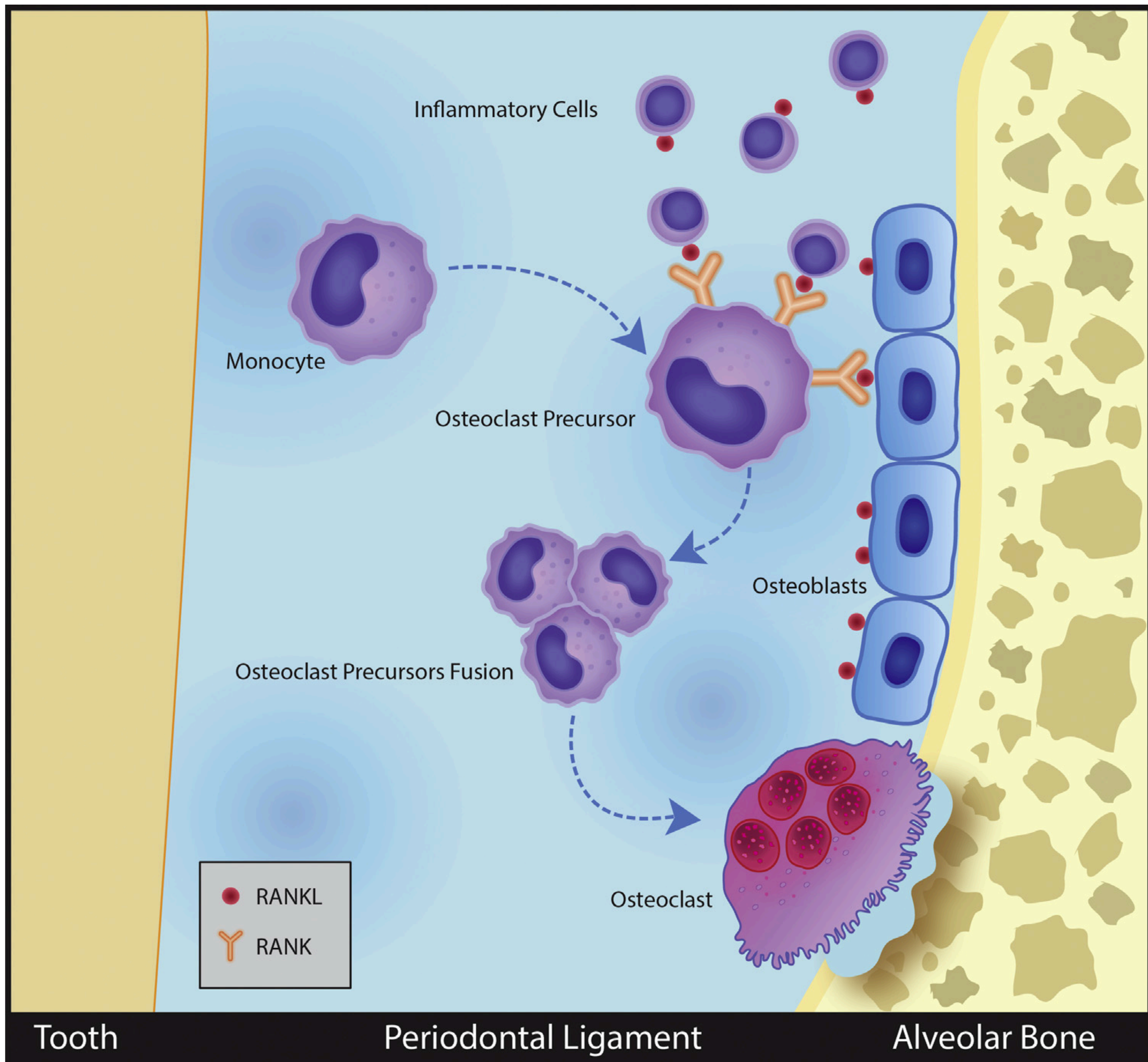


Figure 1-4. Diagram of the effect of cytokines on osteoclastogenesis. Cytokines can directly help in the differentiation or activation of osteoclasts from osteoclast precursor cells. Also, cytokines can stimulate local cells to express RANKL that interacts with its receptor (RANK) on precursor cells and help the development of osteoclasts (Alikhani et al. 2015b).

1.2.2.4. Effect of cytokine inhibition on the rate of tooth movement

The importance of cytokines in controlling the rate of tooth movement can be appreciated from studies that block their effects. It has been shown that injection of IL-1 receptor antagonist or TNF-α receptor antagonist (sTNF-α-RI) results in a 50% reduction in tooth velocity (Andrade et al. 2007; Iwasaki et al. 2001; Jager et al. 2005). Similarly, tooth movement in TNF type II receptor-deficient mice is reduced compared to wild-type mice (Yoshimatsu et al. 2006). Animals deficient in CC chemokine receptor 2 (i.e. the receptor for chemokine ligand 2) or chemokine ligand 3 (CCL3) show a significant reduction in orthodontic tooth movement and the number of osteoclasts (Taddei et al. 2012). Likewise, it is well known that non-steroidal anti-inflammatory (NSAID) drugs can reduce the velocity of tooth movement by inhibiting prostaglandin synthesis (Chumbley and Tuncay 1986; Knop et al. 2012). Inhibition of other derivatives of arachidonic acid, such as leukotrienes, also significantly decreases the rate of tooth movement (Mohammed et al. 1989).

1.2.2.5. Controversy about effect of force magnitude on inflammatory mediators

Taken together, these studies support the conclusion that inflammatory markers play a critical role in orthodontic tooth movement by controlling the rate of osteoclast formation and, therefore, bone resorption. It logically follows that increasing the magnitude of orthodontic forces would trigger a cascade of increased inflammatory marker expression and osteoclastogenesis resulting in faster tooth movement. Surprisingly, one of the biggest controversies in the biology of tooth movement literature revolves around the relation between magnitude of force and the rate of tooth movement. While some studies show that higher forces

do not increase the rate of tooth movement (Quinn and Yoshikawa 1985; Ren et al. 2004), others argue the opposite (Yee et al. 2009). This paradox is explained by the inappropriate use of tooth movement as a measure of the effect of force magnitude on the rate of tooth movement. Although tooth movement is indeed the desired result of the biological response to force, it does not precisely measure the relation between force magnitude and the biological response that causes tooth movement.

Many factors affect the amount of tooth movement independent of the force magnitude. These factors can be intrinsic, such as differences in root and alveolar bone shape or bone density, or they may be extrinsic, such as occlusal forces, chewing habits, or limitation of the mechanical design. These variables are difficult to accurately assess in humans due to the need for 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 study duration, measuring tooth movement as the sole representative of the effect of force magnitude can still produce conflicting results because the biological response varies throughout the stages of tooth movement. Different investigators may capture different stages of this biological response and make erroneous conclusions that are not representative of the complete process.

Because of experimental design limitations mentioned above, it is more logical to study the biological response to different force magnitudes in rats that share a similar genetic background, and use molecular and cellular changes, rather than the amount of tooth movement, as the outcome measurements. The controversy about the effect of magnitude of force on OTM will be address by our rat study in Chapter 4 in this dissertation, as our ***Specific Aim 1*** of this research.

1.2.2.6. Effect of stimulation of cytokines on the rate of tooth movement

If inhibiting inflammatory markers decreases the rate of tooth movement, it is logical to assume that increasing their activity should significantly increase the rate of tooth movement. Indeed, injecting PGs into the PDL in rodents increases the number of osteoclasts and the rate of tooth movement (Kale et al. 2004). Systemic application of misoprostol, a PGE_1 analog, to rats undergoing tooth movement for 2 weeks, significantly increases the rate of tooth movement (Sekhavat et al. 2002). Similarly, local injection of other arachidonic acid derivatives, such as thromboxane and prostacyclin (Gurton et al. 2004), increases the rate of tooth movement.

Another approach to increasing inflammatory mediators that can improve the rate of tooth movement is to stimulate the body to produce these factors at a higher level. The advantage of this approach is a coordinated increase in the level of all inflammatory mediators. As discussed before, many cytokines participate in response to orthodontic forces. Injecting one cytokine does not mimic the normal inflammatory response, which is a balance of pro- and anti-inflammatory mediators. Rather, it exaggerates uncoupled activation of localized cells to resorb or form bone in ways that do not mimic the natural coupled cellular responses to orthodontic forces. However, which approach safely triggers the body to produce higher levels of inflammatory mediators is not clear.

Animal studies have shown that introducing small perforations in the alveolar bone (micro-osteoperforations; MOPs) during orthodontic tooth movement can significantly stimulate the expression of inflammatory mediators (Teixeira et al. 2010). This response is accompanied by a significant increase in osteoclast number, bone resorption and localized osteopenia around all adjacent teeth, which could explain the increase in the rate and magnitude of tooth movement. One may argue that the effects of the shallow MOPs on tooth movement are not a response to

increased cytokine expression, but rather due to weakening of the bone structure. While the effects that perforations can have on the physical properties of the bone cannot be ignored, the number and diameter of these perforations is too small to have significant impact. Similarly, a human clinical trial using a canine retraction model, demonstrates that MOPs can amplify the catabolic response to orthodontic forces. Canine retraction in the presence of MOPs results in twice as much distalization compared with patients receiving similar orthodontic forces without MOPs. This increase in tooth movement is accompanied by an increase in the level of inflammatory mediators (Alikhani et al. 2013).

Clinical studies demonstrate that increasing the number of MOP's significantly increases expression of inflammatory mediators and the magnitude of tooth movement (Alikhani et al. 2015a). Therefore, one should expect procedures such as orthognathic surgery, corticotomies (where a flap is raised and numerous cuts and perforations are made in the alveolar bone), or piezocision (where no flap is raised, and bone is accessed through small cuts through the gingiva, followed by bone injury by a piezoelectric device) to significantly increase the levels of inflammatory cytokines beyond those induced by MOPs. While increase in cytokine release by these methods is accompanied with higher rate of tooth movement, unfortunately, the increase in the expression of inflammatory mediators is not sustained for a long time. A significant decrease in cytokine activity is observed 2-3 months after any of these treatments. As a result, each of these procedures would need to be repeated during the course of orthodontic treatment, which renders some of the above-mentioned modalities impractical.

1.2.3. Anabolic phase of orthodontic tooth movement — Osteoblast activation

The catabolic phase of tooth movement that we just discussed is followed by an anabolic phase that allows the bone to keep its new morphological relation with adjacent structures. Importantly, the anabolic phase must involve both the trabecular and cortical bone. However, the molecular events that initiate the anabolic phase are not clear.

Alveolar bone in the area opposite to the direction of tooth movement is exposed to tensile stresses. Similar to activation of osteoclasts in compression side, the activation of osteoblasts in the tension side cannot be denied. But why are osteoblasts activated in the tension side? Some have suggested that osteoblast activation in these areas is simply a response to tensile stresses. However, many observations discredit this view. While some *in vitro* experiments demonstrate osteoblasts activation in response to tensile forces (Ikegame et al. 2001), these experiments have not been supported by *in vivo* studies. Experiments in long bones and alveolar bone demonstrate that at physiologic levels, osteocyte activation requires intermittent loads of specific frequency and acceleration (Alikhani et al. 2012; Alikhani et al. 2015d; Garman et al. 2007; Rubin et al. 2001). Therefore, application of static tensile forces such as orthodontic forces would not be able to explain bone formation in the tension side. Furthermore, it has been shown that static tensile forces in long bones can cause bone resorption and not formation (Bassett 1968), while under high frequency and acceleration, tensile forces similar to compression forces both can be osteogenic (Hert et al. 1969; Rubin and Lanyon 1984). Thus, other factors should explain the anabolic phase of orthodontic tooth movement.

1.3. Difference in Biological Response among Individuals

Since biological response plays a central role in controlling orthodontic tooth movement, different biological response can result in different amount of tooth movement. The sequence of cellular, molecular, and tissue-reaction events during orthodontic tooth movement has been studied previously (Krishnan and Davidovitch 2006). However, current literature has unclear information on the effect of same orthodontic mechanical stimulus on the biological responses among different individuals. Animal studies have shown that even with standardized, constant, and equal forces, the rate of orthodontic tooth movement can vary substantially among and even within subjects (Pilon et al. 1996; van Leeuwen et al. 1999). Therefore it was concluded that the rate of tooth movement is based mainly on patient characteristics. Several individual factors, alone or in combination, might influence biological response to orthodontic force and ultimately tooth movement. In this regard, age, drug consumption, diet, certain systemic conditions, bone density, tooth morphology, and other intrinsic genetic factors, have been shown to influence the rate of tooth movement (Dudic et al. 2013; Krishnan and Davidovitch 2009; Ren et al. 2003a).

Previous studies have shown that age can play a role the rate of tooth movement. Several studies in animals (Bridges et al. 1988; Kyomen and Tanne 1997) and humans (Giannopoulou et al. 2015; Iwasaki et al. 2005; Kawasaki et al. 2006; Ren et al. 2002) with different force magnitudes, regimens, appliances and observation period have shown that juveniles tend to have greater rate of tooth movement. However, as we discussed in previous section in this Chapter, although tooth movement is the desired result of the biological response to orthodontic forces, it may not necessarily be a precise representative of the biological response that cause tooth movement, since many other factors can affect the amount of tooth movement (Dudic et al. 2013; Krishnan and Davidovitch 2009; Ren et al. 2003a). While majority of these studies focused on

evaluating the difference in the rate of tooth movement, little evidence is available in regards to the differences between different age groups in terms of their biological responses to orthodontic force, especially in human beings. Previous studies comparing biological responses in GCF in different age populations lack consistency in appliance design, protocols, magnitude of force, type of tooth movement, observation period, and biomarkers evaluated (Iwasaki et al. 2005; Kawasaki et al. 2006; Ren et al. 2002; Rody et al. 2014). Therefore this field warrants further research and it will be addressed it in Chapter 5 of this dissertation, as our ***Specific Aim 2*** of this research.

The difference in biological response among different age groups in response to orthodontic force has been related to bone density or rate of osteoclast recruitment or activation (Bridges et al. 1988; Kyomen and Tanne 1997; Ren et al. 2005; Ren et al. 2003b). Increasing bone and mineral densities have been observed as individuals mature (Bridges et al. 1988; Burnell et al. 1980), and therefore faster movement in younger individuals has been partly attributed to lower bone and mineral densities in young bone tissue (Pilon et al. 1996; Reitan 1967). Besides, while some authors argued that number of osteoclasts appears to be higher in younger rats than adult rats (Ren et al. 2005) in the early stage of orthodontic movement, others argued that the number, size and activity of osteoclasts in mechanically stressed alveolar bone during orthodontic tooth movement is the same in young and old rats (Kabasawa et al. 1996).

1.4. Effect of Force Magnitude in Periodontal Tissue Response

Different types of histological changes in periodontium to orthodontic force have been described in literatures. In general, heavier force cause more extensive tissue injury than light forces — light force evoke frontal resorption of bone, while heavy forces often cause necrosis (hyalinization) of the PDL and undermining bone resorption, and have been implicated in root resorption (Krishnan and Davidovitch 2015; Proffit 2013) .

When light force is applied to a tooth, the tooth moves in its socket and compresses the PDL, causing a decrease in blood flow in a few seconds. Within a few hours at most, the resulting change in the chemical environment produces cause aseptic inflammatory response that is discussed earlier in this Chapter. The inflammatory response recruits osteoclasts to the area which attack the adjacent lamina dura and remove bone adjacent to the compressed PDL, producing "**frontal bone resorption**" (Fig. 1-8).

When applying heavier force to a tooth, PDL is compressed to the point that blood flow is totally occluded, causing a sterile necrosis within the compressed area, making differentiation of osteoclasts within the PDL space impossible. Instead, osteoclasts are recruited and differentiated within adjacent marrow spaces and begin to resorb on the underside of the lamina dura immediately adjacent to the necrotic PDL area. Such process is called "**undermining bone resorption.***"* The histologic appearance of the necrotic zone, an avascular area in the PDL, is referred to as hyalinized or **cell-free zone**. The remodeling of bone adjacent to the cell-free zone of the PDL has to be accomplished by cells derived from adjacent undamaged areas.

When undermining resorption occurs, there is a delay in stimulating cell differentiation within the marrow space, and it takes longer for the osteoclasts to remove the bone from the marrow space before the tooth can start its movement; therefore, there is a delay in tooth movement when compared with when frontal resorption predominates around the tooth. In reality, it is difficult to avoid pressure that produces at least some avascular areas in the PDL even with light force.

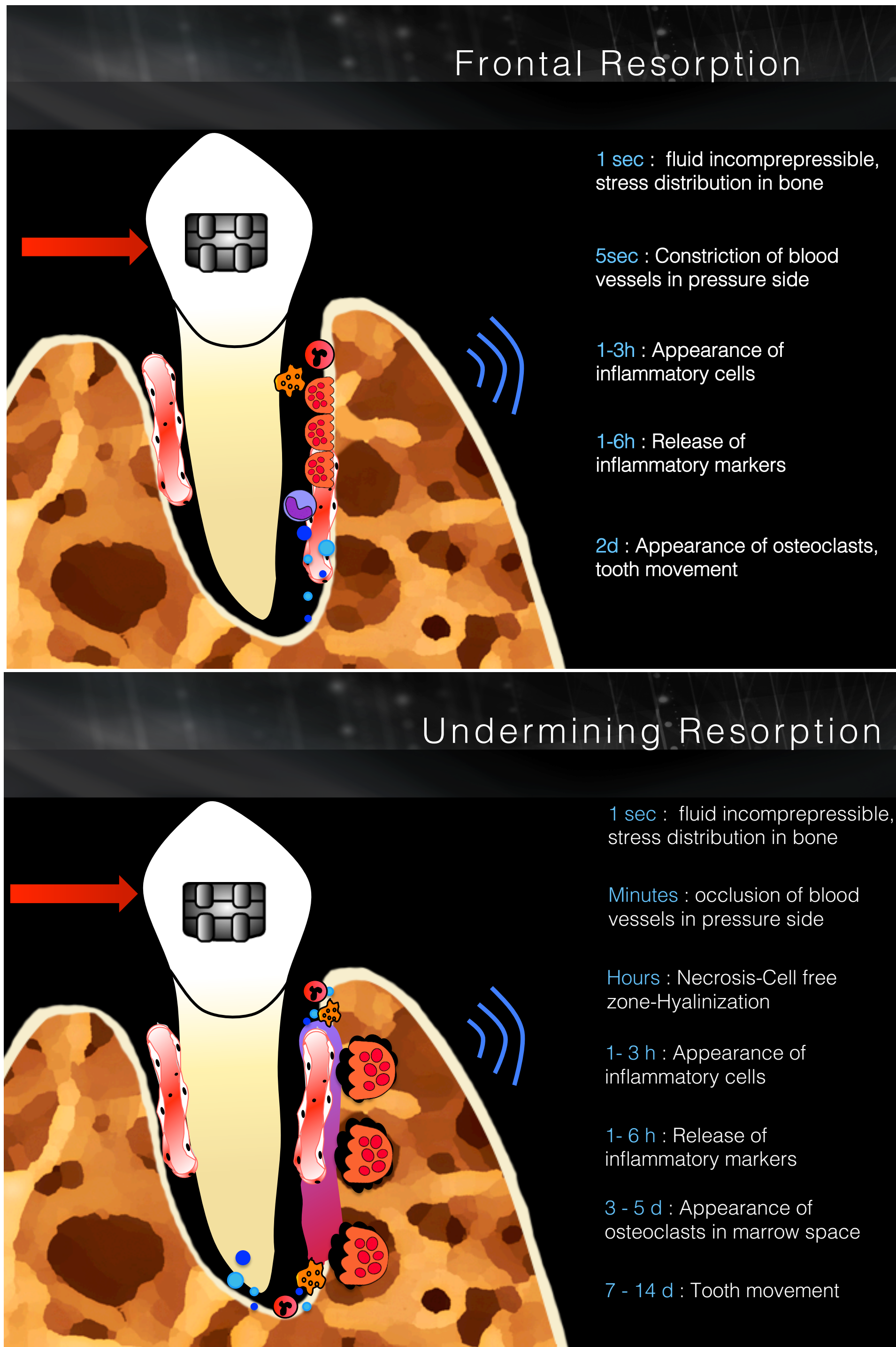


Figure 1-5. Types of physiologic response and its chronological biological events in response to orthodontic force. The diagram shows the course of events in frontal and undermining bone resorption.

Chapter 2. Hypothesis and Specific Aims

The objective of this study is to research the effect of orthodontic force magnitude on cytokine activation, osteoclast formation, and subsequent rate of tooth movement in the short-term and long-term.

The null hypothesis of this study is that there is no difference in the level of cytokine activation within individuals or between individuals receiving different magnitudes of orthodontic forces.

This hypothesis was addressed by the following specific aims:

Specific Aim 1: To investigate the expression and activity of inflammatory markers in response to different magnitudes of orthodontic forces.

- Sub-aim 1A: To investigate the spectrum of inflammatory markers that are activated in response to different magnitudes of orthodontic forces.
- Sub-aim 1B: To investigate the activity of inflammatory markers in response to different magnitudes of orthodontic forces in a longer time period.
- Sub-aim 1C: To investigate osteoclast activity and the rate of tooth movement in response to different magnitudes of orthodontic forces at different time points.

Specific Aim 2: To investigate the effect of age on the activity of inflammatory markers and the correlated rate of tooth movement while keeping force magnitudes equal between groups.

- Sub-aim 2A: To investigate the activity of inflammatory markers in different age groups while keeping orthodontic force magnitude equal between groups.

- Sub-aim 2B: To investigate the rate of tooth movement in different age groups while keeping orthodontic force magnitude equal between groups.

Specific Aim 3: To investigate the activity of inflammatory markers and the correlated effect on the rate of orthodontic tooth movement among individuals of different ages while using different force magnitudes.

- Sub-aim 3A: To investigate the inflammatory marker activity in response to different orthodontic force magnitudes in different age groups.
- Sub-aim 3B: To investigate the rate of tooth movement in response to different orthodontic force magnitudes in different age groups.

Chapter 3. Research Strategy: Significance, Innovation, and Approach

3.1. Significance

Increasing the rate of tooth movement in a safe manner to reduce the duration of orthodontic treatment would have a significant impact on a patient's oral health and social life (Bernabe et al. 2008; Jones and Chan 1992; Liu et al. 2011; Ngan et al. 1989; Oliver and Knapman 1985; Proffit and Sarver 2013; Sergl et al. 1998; Zhang et al. 2008). Shorter treatment duration is usually desirable to orthodontists and patients because treatment duration has been associated with an increased risk of gingival inflammation (Ristic et al. 2007), decalcification (i.e. white spot lesions), dental caries (Huang et al. 2013; Julien et al. 2013; Oosterkamp et al. 2016), and, especially, root resorption (Segal et al. 2004). One of the frequently asked questions by patients or their parents in orthodontists' daily practice is, "When can we take the braces off?" It is not uncommon orthodontists encounter requests from, especially adult patients, to make the duration of treatment as short as possible, or to take off braces sooner than expected due to unforeseen changes in their personal lives. Prolonged treatment time not only increasing burden on patients and their caregivers, but also can result in pathological changes such as root resorption (Fox 2005; Roscoe et al. 2015; Sameshima and Sinclair 2001; Segal et al. 2004; Uribe et al. 2014; Weltman et al. 2010).

At individual level, a recent survey showed that more than 33.3% of adult patients, 54.8% of adolescent patients and 25.2% of parents thought their orthodontic treatment took too long. Most adolescents (40.8%) desired their orthodontic treatment to last less than six months, and most adults (42.9%) desired 6 to 12 months (Uribe et al. 2014). There is a huge gap between the desired duration of treatment and the reality–average treatment duration of 23.8 months for non-

extraction case and 28.1 months for extraction cases (Buschang et al. 2012; Fink and Smith 1992; Uribe et al. 2014). In fact, more than 60% of parents and adult patients are positive or neutral to pay higher treatment fee for reducing treatment time (Uribe et al. 2014). Furthermore, at the societal level, considering that greater than 2.6 million people worldwide start orthodontic treatment each year (Align Technology 2014), and that greater than 5.41 million patients in active treatment in USA and Canada in 2014 (American Association of Orthodontists 2015), the positive cumulative impact of shortening the length of orthodontic treatment is apparent.

Since force application initiates tooth movement, it seems logical to clinicians to assume that increasing the magnitude of force should increase the rate of tooth movement; however, this assumption is currently unproven. If this assumption is not true, it may lead clinicians to apply additional force whenever they do not obtain the expected rate of tooth movement at the expense of patient comfort and dental health. Furthermore, greater force magnitudes have been associated with greater intensity of pain and discomfort (Luppanapornlarp et al. 2010), and an increased risk of root resorption (Nakano et al. 2014; Proffit 2013; Roscoe et al. 2015; Weltman et al. 2010).

In addition, current literature has scarce information on the effect of individual variability on the biological and clinical outcomes to mechanical stimulations, and through what mechanism do these individual factors play a part. Several studies have shed light on the effect of individual factors on the rate of tooth movement and its plausible mechanism (Dudic et al. 2013; Krishnan and Davidovitch 2009; Ren et al. 2003a), however, due to heterogeneity of these studies, a well-round perspective on this subject has yet to be carried out. Since most orthodontic appliances, such removable or edgewise appliances, are not quite designed to take individual variations (either at patient or individual tooth level) into account, if individual factor does play a significant role in biological response to orthodontic stimuli, then our current treatment system

warrants significant changes and improvement to provide our patients more optimized and efficient treatment.

In summary, understanding the mechanism through which force magnitude affects the rate of tooth movement will help reveal biological targets that could potentially be stimulated, either directly or indirectly, to achieve faster and safer treatment without increasing the magnitude of force. In addition, understanding the effect of individual variations on the biological response to orthodontic force will help clinicians to make optimized and customized treatment decisions for their patients, and improve the treatment efficiency by modifying the current treatment system and modalities.

3.2. Innovation

Current literature has shown limited and contradictory results in terms of the relation between magnitude of orthodontic force and the rate of tooth movement, within or among different individuals. This field has received surprisingly little attention and very few experimental or clinical studies have been performed. It is currently unproven that increasing the magnitude of force results in an increase in the rate of tooth movement. Interestingly, even fewer number of studies explored the relation between the magnitude of orthodontic force and the biological responses that cause OTM. Understanding such relation is essential since the rate of OTM is not a precise representative of the level of biological response, as we discussed in previous Chapter. In addition, the difference in biological responses among individuals is yet to be clarified. Therefore, we proposed a series of studies to address these research questions. We believe by understanding these fundamentals, we will eventually be able to improve the efficiency and safety of orthodontic treatment by optimizing the current treatment system, and furthermore, potentially reveal essential biological targets that could potentially be stimulated and advance methodologies to accelerate tooth movement.

These studies are innovative because it is the first research that emphasized to use molecular and cellular changes as comparative parameters instead of solely amount or rate of tooth movement, in response to an equal or different magnitude of orthodontic force within or among individuals. We also, for the first time, closely evaluated the amount of biological response and its "saturation" in molecular, cellular, and clinical levels. Such biological response can be different among individuals, and therefore "saturation point" may vary among individuals.

3.3. Clinical Relevance

This research is to address one of the fundamental clinical questions in orthodontics, which is the effect of orthodontic force magnitude on the rate of tooth movement. Since there is an increasing demand by patients to accelerate tooth movement in order to shorten treatment duration, how to achieve such goal without compromising patients' dental health is essential. Therefore, clinicians need to understand the effect of different magnitude of force and rate of tooth movement, in the same individual and among different individuals, in order to ultimately utilize the current treatment system to an optimal level. Clinicians also need to be aware of any side effect on their patients' periodontal health resulted from more-than-necessary amount of force.

If increasing magnitude of force cannot further increase the rate of tooth movement, then higher forces to increase the rate of tooth movement are not justified, and other methods should be considered during orthodontic tooth movement. Therefore, this study also provides a foundation for understanding the rationale and necessity for accelerated orthodontic techniques.

3.4. Approach

Since 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 expression of inflammatory markers and, therefore, rate of tooth movement. In the following three chapters, three studies in both animals (rat) and humans were elaborated, in order to research the effect of orthodontic force magnitude on cytokine activation, osteoclast formation, and subsequent rate of tooth movement in the short-term and long-term. The first study was done on a rat model to investigate the saturation of biological responses to increased magnitude of force, as if it was in the same individual. The second study was done on in humans with different ages, to investigate different inflammatory responses to an equal magnitude of force in different individuals, using their age as the main differentiating factor. The third study was done in humans with different ages, to investigate the difference in saturation of biological responses among individuals.

3.4.1. Approach to Specific Aim 1

Our first aim is to investigate the expression and activity of inflammatory markers in response to different magnitudes of orthodontic forces. 245 Sprague Dawley male rats were divided into control, sham, and different experimental groups. Experimental groups received different magnitudes of force (3 to 100 cN) to the maxillary right first molar using a Sentalloy coil spring. In the sham group the spring was not activated. Control group did not receive any appliance. At different time points, the maxillae were collected for RNA and protein analysis, in order to investigate the spectrum of inflammatory markers that are activated in response to

different magnitudes of orthodontic forces, and to investigate the activity of inflammatory markers in response to different magnitudes of orthodontic forces in a longer time period. To correlate the molecular response with cellular and clinical responses, we further studied the histological sections of mesial aspect of mesiopalatal root of maxillary first molars at different time points to investigate the cellular reaction and patterns of bone resorption. Osteoclast activity in response to different magnitudes of orthodontic forces at different time points was studied using immunohistochemistry. Lastly, the rate of tooth movement in response to different magnitudes of orthodontic forces at different time points was studied using micro CT scanned images.

3.4.2. Approach to Specific Aim 2

Our second aim is to investigate the expression and activity of inflammatory markers and its correlated rate of tooth movement in response to an equal magnitude of orthodontic force among different individuals, using their "age" as the differentiating variable. A non-randomized, single-blinded clinical study was designed to recruit healthy human subjects in both sexes and in different age groups (age 11-14 and 21-45). Patients were recruited based on 1) meeting the inclusion and exclusion criteria and 2) needing maxillary canine retraction of at least 3 mm. All subjects were monitored for oral hygiene and all received a constant force of 50 cN produced by a Sentalloy coil spring hooked from the ipsilateral first molar. The force was applied close to the center of resistance of the canine to produce bodily retraction. To investigate the activity of inflammatory markers in different age groups, gingival crevicular fluid (GCF) from distolabial crevice of canines were collected, and the activity of different inflammatory markers in GCF including IL-1β, CCL2, RANKL, and MMP-9 were measured using antibody-based assays at different time points: baseline (before orthodontic treatment), immediately before canine retraction, 1, 7, 14, and 28 days after the canine retraction was initiated. To investigate the rate of tooth movement in different age groups, dental study models of the maxillary arch at 28 and 56 days after retraction were measured and compared with pre-retraction. Significant differences between groups were assessed by analysis of variance (ANOVA). Pairwise multiple comparison analysis was performed with the Tukey's *post hoc* test.

3.4.3. Approach to Specific Aim 3

Our third aim is to investigate the saturation of biological response to orthodontic forces and its correlated rate of orthodontic tooth movement among different individuals, using their age as the base for variability among individuals. A non-randomized, single-blinded clinical study was approved by IRB. Healthy human subjects in both sexes and in different age groups (age 11-14 and 21-45) were recruited. Patients were recruited based on 1) meeting the inclusion and exclusion criteria and 2) needing maxillary canine retraction of at least 3 mm. All subjects were monitored for oral hygiene, and were assigned to receive different magnitudes of force produced by a Sentalloy coil spring hooked from the ipsilateral first molar: 50, 100, 150 and 200 cN. The force was applied close to the center of resistance of the canine to produce bodily retraction. To investigate the inflammatory marker activity in response to different orthodontic force magnitudes in different age groups, gingival crevicular fluid (GCF) from distolabial crevice of canines were collected one day after retraction. The activity of different inflammatory markers in GCF including IL-1β, CCL2, and RANKL were measured using antibody-based assays. To investigate the rate of tooth movement in response to different orthodontic force magnitudes in different age groups, dental study models of the maxillary arch at 28 days after retraction were measured and compared with pre-retraction. Significant differences between groups were assessed by analysis of variance (ANOVA). Pairwise multiple comparison analysis was performed with the Tukey's *post hoc* test.

Chapter 4. Saturation of the biological response to orthodontic forces and its effect on the rate of tooth movement: An animal study

This Chapter focused on research strategy and results to our Specific Aim 1, which is to investigate the expression and activity of inflammatory markers in response to different magnitudes of orthodontic forces.

- Sub-aim 1A: To investigate the spectrum of inflammatory markers that are activated in response to different magnitudes of orthodontic forces.
- Sub-aim 1B: To investigate the activity of inflammatory markers in response to different magnitudes of orthodontic forces in a longer time period.
- Sub-aim 1C: To investigate osteoclast activity and the rate of tooth movement in response to different magnitudes of orthodontic forces at different time points.

4.1. 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.

Methods and Materials: 245 Sprague Dawley male rats were control, sham, and different experimental groups. Experimental groups received different magnitudes of force to the right maxillary first molar using a Sentalloy coil spring. In the sham group the spring was not activated. Control group did not receive any appliance. At different time points, 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 during lower magnitudes of force. Higher magnitudes of force did not increase the expression of cytokines. Activity of CCL2, CCL5, IL-1, TNF, RANKL, and number of osteoclasts reached a saturation point in response to higher magnitudes of force long term, which was accompanied by unchanged rate of tooth movement.

Conclusion: After a certain magnitude of force, there is a saturation in the biological response, where higher magnitude of force does not increase inflammatory markers, osteoclasts, nor 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

4.2. Introduction

Tooth movement occurs in response to orthodontic forces. However, this movement is not completely regulated by the law 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 (Iwasaki et al. 2009; Krishnan and Davidovitch 2006) 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 (Krishnan and Davidovitch 2006). These release more inflammatory markers that directly or indirectly—through mediators such as prostaglandins—activate RANK-RANKL pathway, stimulating osteoclast precursor cells into osteoclasts (Yamaguchi 2009). The importance of cytokines can be appreciated in experiments in which inhibition of inflammatory markers blocks orthodontic tooth movement (Arias and Marquez-Orozco 2006; Knop et al. 2012).

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 the rate of tooth movement. Increasing the cytokine release by applying minor trauma to the alveolar bone can significantly increase the number of osteoclasts and rate of tooth movement in both animals and humans (Alikhani et al. 2013; Teixeira et al. 2010). 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

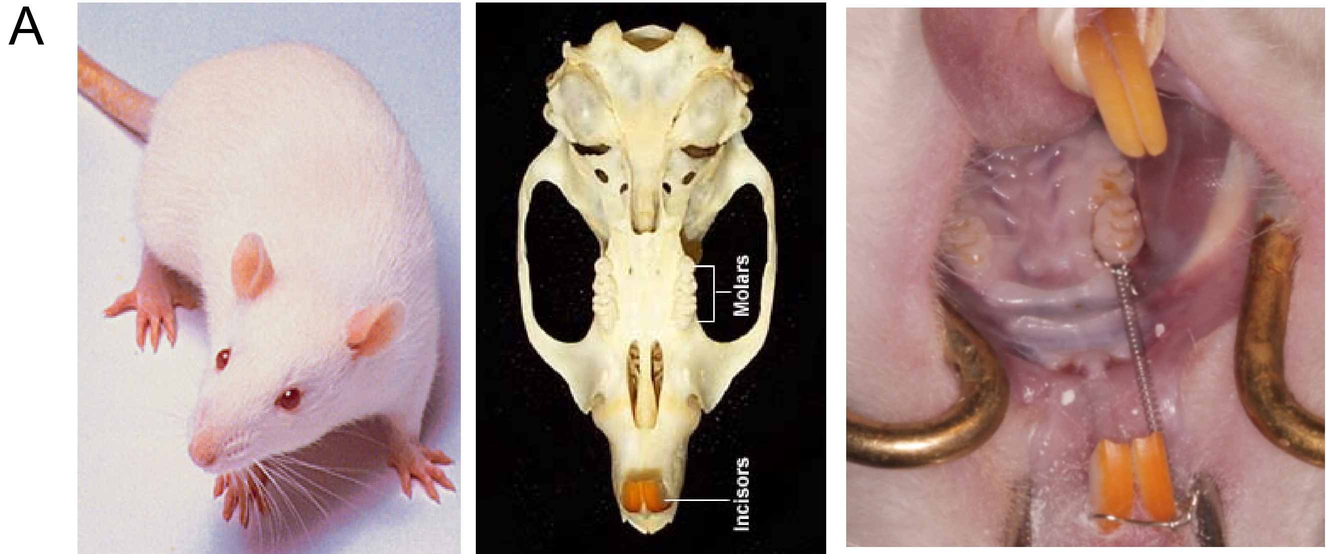
to increase the rate of tooth movement would be justified. But if this assumption is not true, then application of higher forces does not have any clinical advantage and only exposes patients to higher risk of side effects such as prolonged hyalinization and root resorption (Chan and Darendeliler 2005).

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.

4.3. Materials And Methods

4.3.1. Animal Study

Sprague Dawley rats (245 adult males: average body 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 without activation (0 cN), and control group did not receive any treatment (Fig. 4-1). Sentalloy closing coils (GAC International, Bohemia, NY, USA) were designed so that 1 mm activation provided required force. All coil springs were calibrated at 37 degrees with certified digital force gauge (Phase II Plus, Upper Saddle River, NJ, USA) to ensure consistency and reproducibility of the force. Springs were checked daily without reactivation during the experimental period using inhalation anesthesia (isoflurane). Animals with loose spring were excluded. After force application, the specimens were collected at days 1, 3, 7, 14, and 28 days (five animals per group, per time point). Procedures were performed on one side of the maxilla, allowing the contralateral side to be used as internal control.



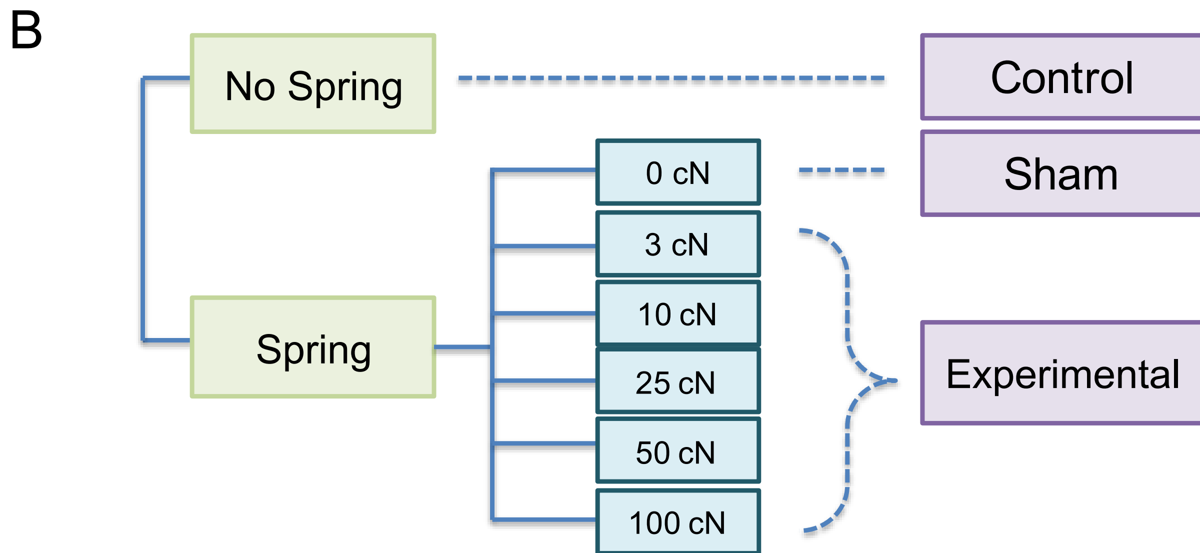


Figure 4-1. (A) Anatomy of maxilla of rats and the appliance used in this animal study, demonstrating positions of incisors and molars. Calibrated Sentalloy coil springs were ligated on right maxillary incisor and first molar, either inactive or applying different magnitudes of force ranged from 3 cN to 100 cN. **(B)** Diagram of the study design and group assignment.

4.3.2. 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 of the teeth (Fig. 4-2). 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 (Dahlberg 1940a). 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).

Figure 4-2. Micro-CT images were used to evaluate the rate of tooth movement. 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 of the teeth. The average distance between those points was calculated to quantify tooth movement.

4.3.3. 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 a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). As negative control, sections were exposed to pre-immune serum. Osteoclasts were defined as Cathepsin K–positive multinuclear cells on periosteal or endosteal bone surface 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.

4.3.4. RNA Analysis

For RNA extraction, five animals from each group were sacrificed by CO_2 narcosis at 24 hours, and the hemi-maxillae were dissected and frozen in liquid nitrogen. Isolation of total RNA was performed as described previously (Oliveira et al. 2009). 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). 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.

4.3.5. Protein Analysis

Activity of different inflammatory markers was measured by enzyme-linked immunosorbent assay (ELISA). Five hemi-maxillae 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-□) (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 to standard curves specific to each inflammatory marker.

4.3.6. 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.

4.4. Results

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

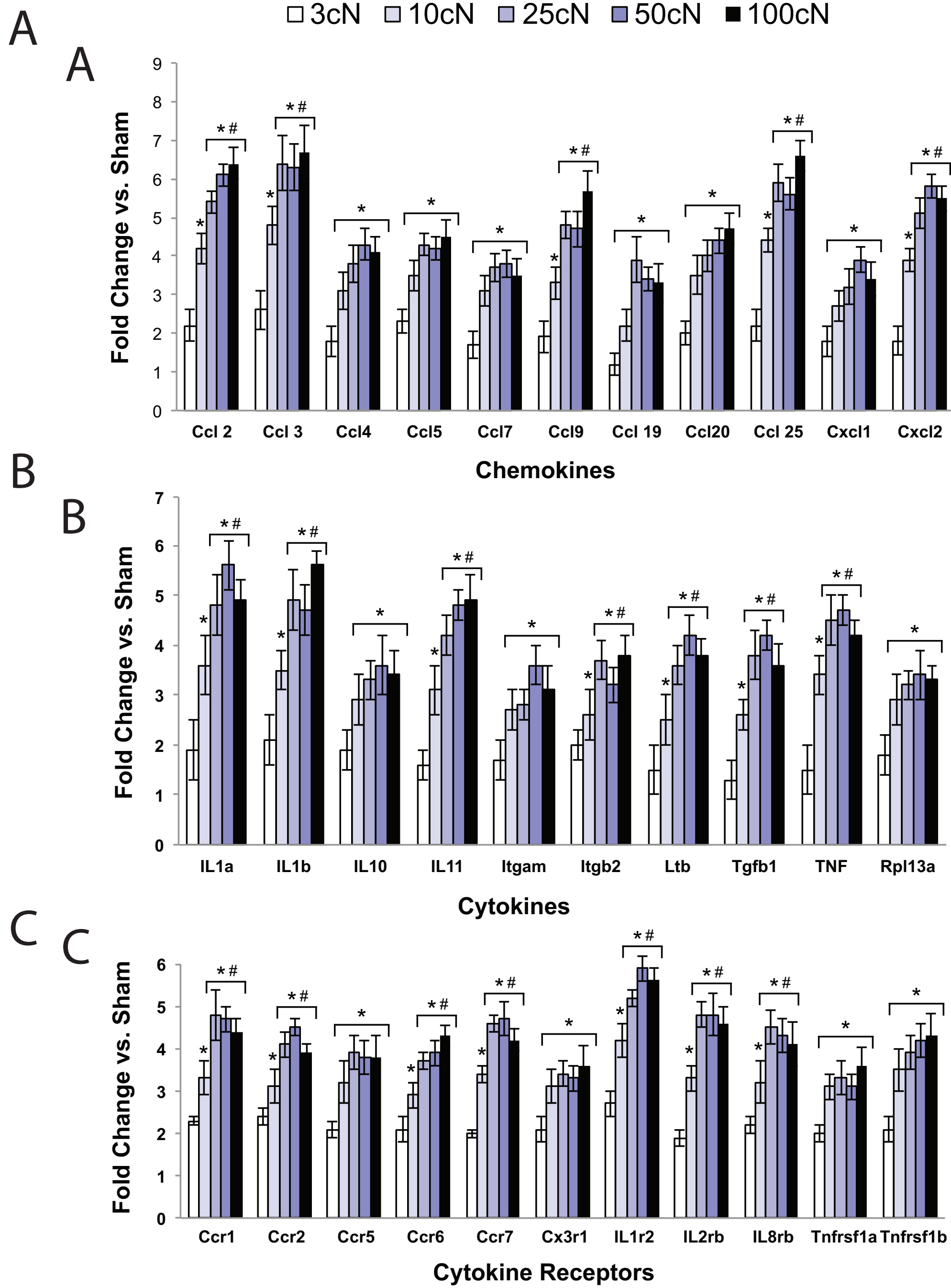
Expression of 86 different cytokines, chemokines, and their receptors was evaluated 24 hours after application of different force levels. In comparison with sham group, the expression of 32 chemokines (Fig. 4-3, A), cytokines (Fig. 4-3, B), and their receptors (Fig. 4-3, C) increased more than twofold in experimental animals. The range of expression was 1.3- to 2.7-fold in 3 cN group, 2.2- to 4.8-fold in 10 cN group, 2.8- to 6.4-fold 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 cN, 50 cN, 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. 4-3, D), CCL5 (Fig. 1, E), IL-1 (Fig. 4-3, F), and TNF-α (Fig. 4-3, G) 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 ($p < 0.05$).

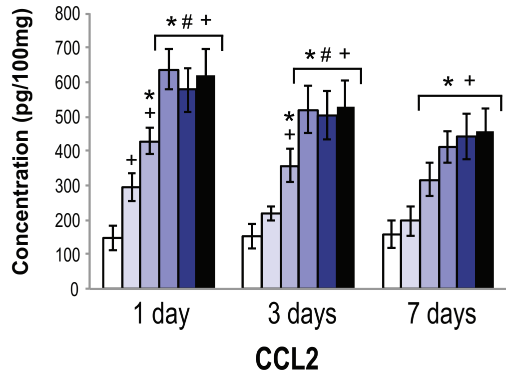
IL-1 concentration decreased on days 3 and 7 for all groups but was still significantly higher than sham group ($p < 0.05$) except for the 3 cN group at day 7. No differences in CCL2,

CCL5, and IL-1 were observed between 25, 50, and 100 cN groups at any time point ($p > 0.05$).

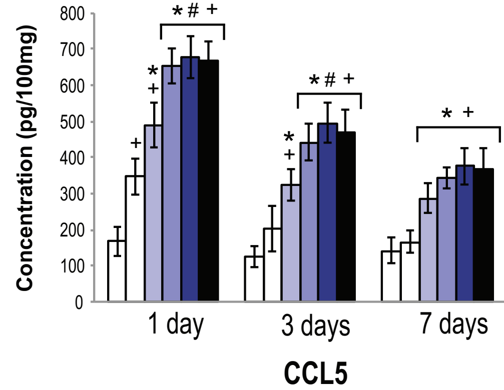
TNF-α concentration showed no difference from 10 to 100 cN of force at day 1 ($p > 0.05$). At days 3 and 7 for all groups, the concentration of TNF-α significantly decreased and no statistical differences were observed between sham and experimental groups at those time points ($p > 0.05$).

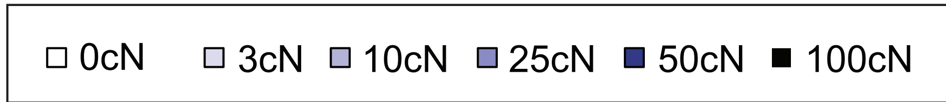

A
B
C
3cN
10cN
25cN
50cN
100cN
Fold Change vs. Sham
Ccl 2
Ccl 3
Ccl4
Ccl5
Ccl7
Ccl9
Ccl 19
Ccl20
Ccl 25
Cxcl1
Cxcl2
Chemokines
IL1a
IL1b
IL10
IL11
Itgam
Itgb2
Ltb
Tgfb1
TNF
Rpl13a
Cytokines
Ccr1
Ccr2
Ccr5
Ccr6
Ccr7
Cx3r1
IL1r2
IL2rb
IL8rb
Tnfrsf1a
Tnfrsf1b
Cytokine Receptors

D

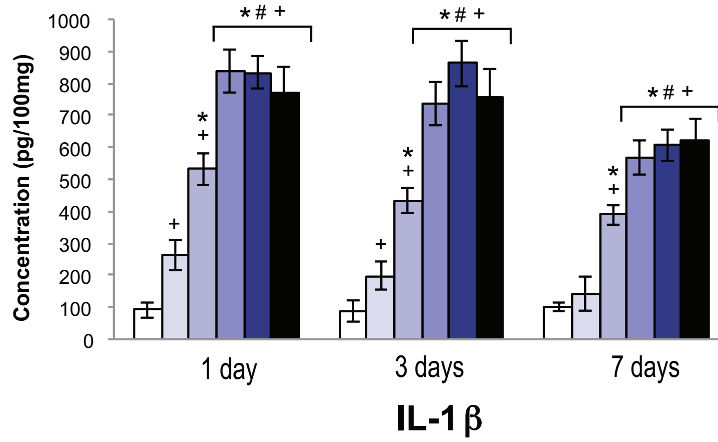


E





F



G

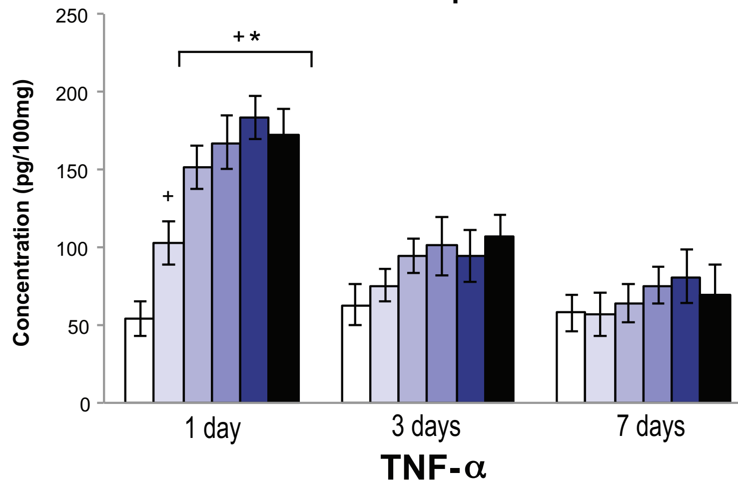


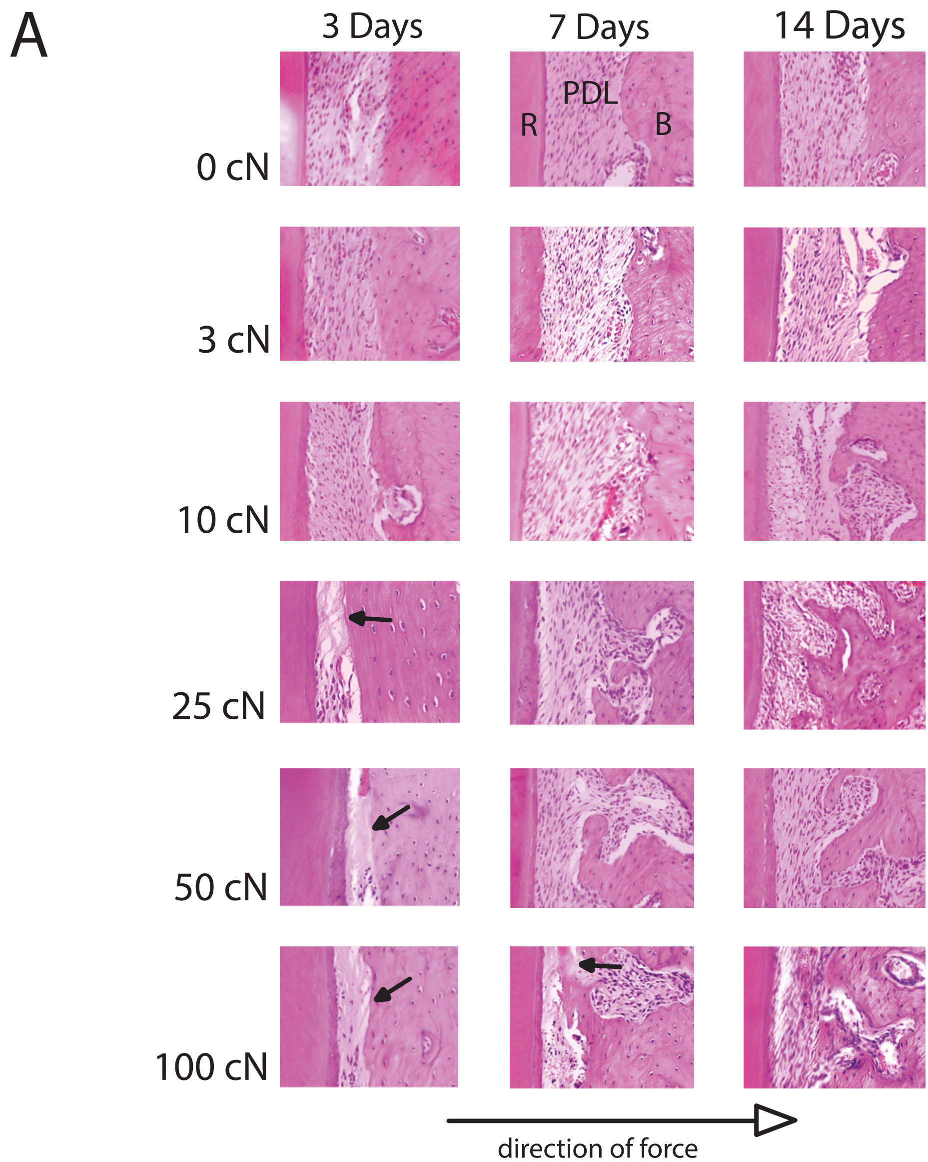
Figure 4-3. Cytokines and chemokines demonstrate saturation in expression and activity in response to higher magnitude of orthodontics force. Mean 'fold' increase in expression of different chemokines **(A)**, cytokines **(B)**, and their receptors **(C)** in force groups was compared with sham group. 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-□ **(G)** in the rat right maxillary alveolar bone after 1, 3, and 7 days of application of different magnitude of the force was evaluated by ELISA. Data expressed as the mean ± SEM 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).

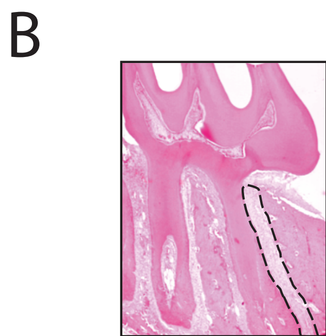
4.4.2. Low and high magnitude of forces produced similar histological changes

We studied the cellular reaction 3, 7, and 14 days after application of different magnitude of orthodontics forces. We evaluated the mesial half of the mesiopalatal root of the first maxillary molar in histological sections. At day 3 (Fig. 4-4, A), 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 groups ($p < 0.05$); no significant differences were observed among 25, 50, and 100 cN groups ($p > 0.05$) (Fig. 4-4, B and C).

Seven days after application of orthodontic forces, all animals presented widening of PDL and areas of bone resorption from both periosteal (frontal resorption) and endosteal side (undermining resorption) (Fig. 4-4, A). 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. 4-4, A).


A
3 Days
7 Days
14 Days
PDL
R
B
0 cN
3 cN
10 cN
25 cN
50 cN
100 cN
direction of force


B

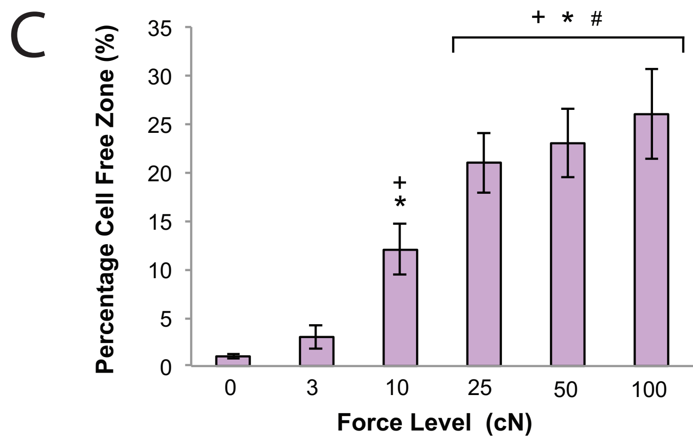

C
Percentage Cell Free Zone (%)
35
30
25
20
15
10
5
0
+ * #
+
*
0
3
10
25
50
100
Force Level (cN)

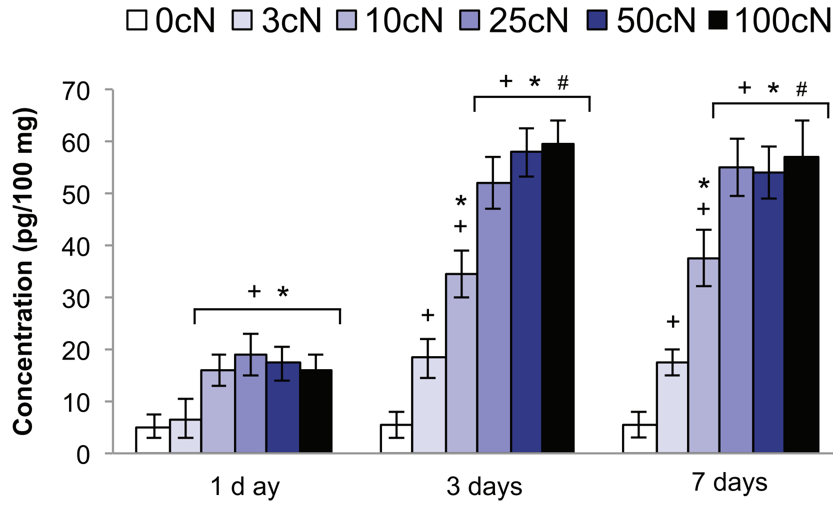
Figure 4-4. Histological changes occurred in response to higher magnitude of orthodontic forces. (A) Light microphotographs of H and E stained sections at days 3, 7, and 14 after application of forces. Area shown corresponds to the mesiopalatal root of upper first molar (*R*), periodontal ligament (*PDL*), and bone (*B*). Areas of high stress close to alveolar crest in the mesial PDL of the root show decreased PDL thickness and larger areas of cell-free zone (*black arrows* at day 3). **(B)** 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 of mesial PDL. **(C)** Each value represents the mean ± SEM of five animals (+, significantly different from 0 cN; *, significantly different from 3 cN; #, significantly different from 10 cN).

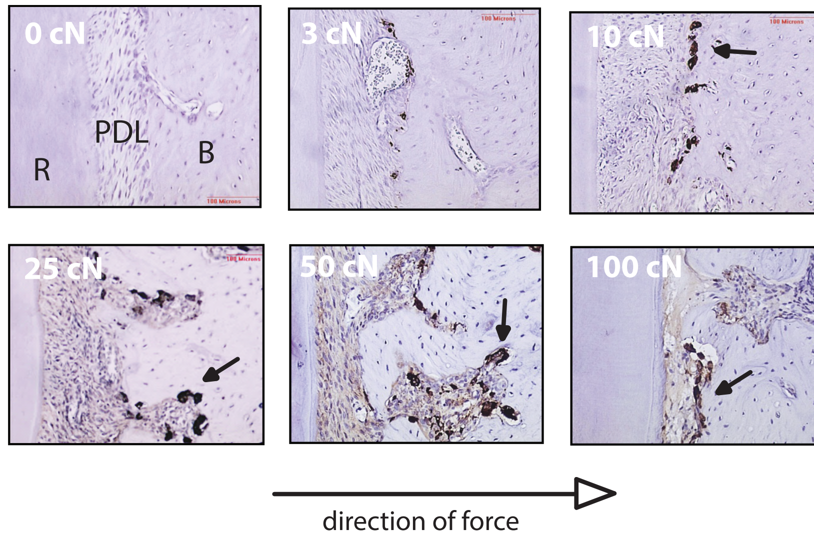
4.4.3. Higher magnitude of force does not stimulate osteoclastogenesis markers or an increase in osteoclasts

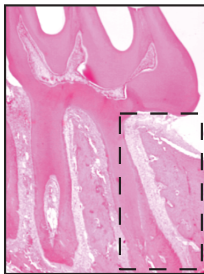
To evaluate the effect of magnitude of force on osteoclastogenesis, we performed ELISA for osteoclast marker RANKL. There was no significant difference between 3 cN and sham groups at day 1 ($p < 0.05$), while the concentration of RANKL in other groups increased 3- to 3.6-fold and was statistically significant ($p > 0.05$). 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 the 3 and 10 cN groups at both time points (Fig. 4-5, A).

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. 4-5, B) or in the apex area in opposite direction of tooth movement. In the 3 and 10 cN groups at day 7, 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. 4-5, C and D). Numbers of osteoclasts were significantly higher in all groups in comparison with sham group ($p < 0.05$).


A
0cN 3cN 10cN 25cN 50cN 100cN
Concentration (pg/100 mg)
70
60
50
40
30
20
10
0
+ *
+ * #
1 d ay
3 days
7 days


B
0 cN
PDL
R
B
3 cN
10 cN
25 cN
50 cN
100 cN
direction of force


C

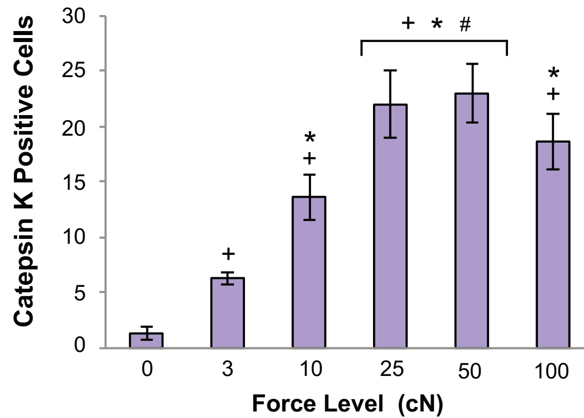

D
Catepsin K Positive Cells
30
25
20
15
10
5
0
+ * #
Force Level (cN)
0
3
10
25
50
100

Figure 4-5. 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 0cN; *, significantly different from 3cN; #, significantly different from 10 cN).

4.4.4. 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 micro-CT, at days 14 and 28. 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 day 14 (Fig. 4-6, A) or 25, 50, and 100 cN force at day 28 (Fig. 4-6,B).

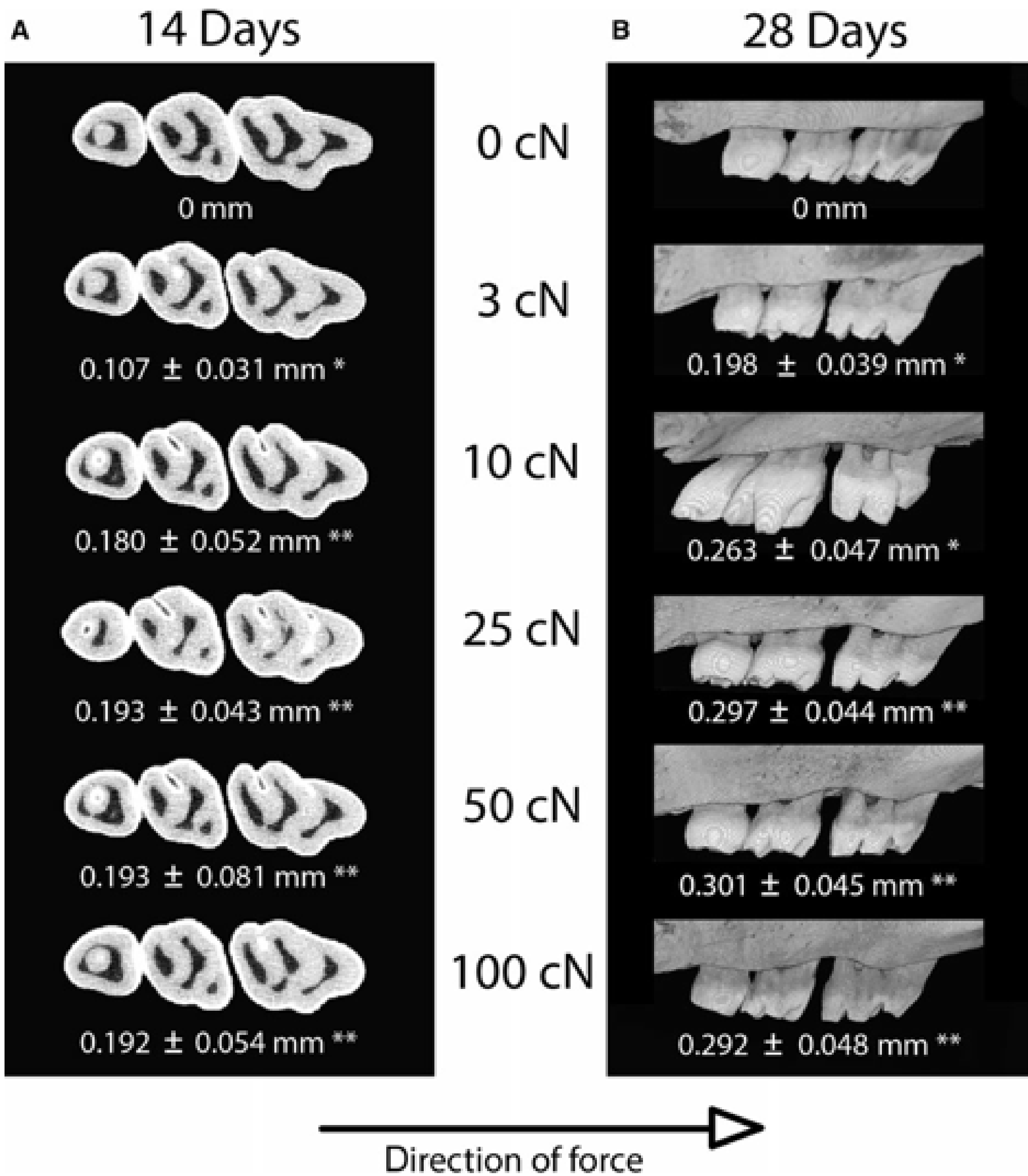

A
14 Days
B
28 Days
0 cN
0 mm
0 mm
3 cN
0.107 ± 0.031 mm *
0.198 ± 0.039 mm *
10 cN
0.180 ± 0.052 mm **
0.263 ± 0.047 mm *
25 cN
0.193 ± 0.043 mm **
0.297 ± 0.044 mm **
50 cN
0.193 ± 0.081 mm **
0.301 ± 0.045 mm **
100 cN
0.192 ± 0.054 mm **
0.292 ± 0.048 mm **
Direction of force

Figure 4-6. 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).

4.5. 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 (Quinn and Yoshikawa 1985; Ren et al. 2004), and others have argued the opposite (Yee et al. 2009). 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 orthodontic forces, it may 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 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, instead of 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 (Isaacson et al. 1993) with minimum stress around the tooth's center of rotation (Smith and Burstone 1984).

At the molecular level, 24 hours after application of different magnitude of forces, the expression of inflammatory markers was stimulated, as seen in previous studies (Alhashimi et al. 2001). 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. The plateau occurred between 10 and 25 cN of force. While we did not investigate forces in between that range, our results established 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 hemi-maxilla, 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 (Tanne et al. 1987). 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 (Nakano et al. 2014).

4.6. Conclusions

1. 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.
2. To increase the rate of tooth movement, the saturation of the biological response must be overcome by other methods.

Chapter 5. Biological response and rate of tooth movement among individuals in response to an identical magnitude of orthodontic force: A clinical study

This Chapter depicted the research strategy and results of our *Specific Aim 2*, which is to investigate the effect of age on the activity of inflammatory markers and the correlated rate of tooth movement while keeping force magnitudes equal between groups.

- Sub-aim 2A: To investigate the activity of inflammatory markers in different age groups while keeping orthodontic force magnitude equal between groups.
- Sub-aim 2B: To investigate the rate of tooth movement in different age groups while keeping orthodontic force magnitude equal between groups.

5.1. Abstract

Objectives: Investigate the activity of inflammatory markers and the correlated rate of tooth movement in response to an equal magnitude of orthodontic force in two age groups.

Methods and Materials: Healthy human subjects in both sexes and in different age groups (age 11-14 and 21-45) were recruited. Canine retraction was rendered with a constant force of 50 cN, and gingival crevicular fluid (GCF) from distolabial crevice was collected at different time points: prior to orthodontic treatment, immediately before initiation of canine retraction, 1, 7, 14, and 28 days after the canine retraction was initiated. The activity of inflammatory markers in GCF including IL-1β, CCL2, TNF-α, RANKL, and MMP-9 were measured using antibody-based assays. The rate of canine retraction in 28 and 56 days was measured on study models. Differences within and between groups were assessed.

Results: Fourteen subjects were recruited with 7 per group. One day after canine retraction, IL-1β, CCL2, TNF-α, RANKL, and MMP-9 increased significantly in both age groups, when compared with the respective level of concentration immediately before canine retraction was initiated. Inflammatory marker activity was significantly higher in adults compared with adolescents. However, the rate of tooth movement was greater in adolescents than adults in the second month of canine retraction, and during the 56-day study period.

Conclusion: Individuals of different ages demonstrated different biological response to an identical magnitude of force. The adults maintained a higher level of biological response, but demonstrated a lower rate of tooth movement. Therefore, one should always compare the level of inflammatory markers to their baseline within the same individual.

5.2. Introduction

Since biological response plays a central role in controlling orthodontic tooth movement (Alikhani et al. 2015b), different biological response can result in different amount of tooth movement. The sequence of cellular, molecular, and tissue-reaction events during orthodontic tooth movement has been studied previously (Krishnan and Davidovitch 2006). However, current literature has unclear information on the effect of same orthodontic mechanical stimulus on the biological responses among different individuals. Animal studies have shown that even with standardized, constant, and equal forces, the rate of orthodontic tooth movement can vary substantially among and even within subjects (Pilon et al. 1996; van Leeuwen et al. 1999). Therefore it was concluded that the rate of tooth movement is based mainly on patient characteristics. Several individual factors, alone or in combination, might influence biological response to orthodontic force and ultimately tooth movement. In this regard, age, drug consumption, diet, certain systemic conditions, bone density, tooth morphology, and other intrinsic genetic factors, have been shown to influence the rate of tooth movement (Dudic et al. 2013; Krishnan and Davidovitch 2009; Ren et al. 2003a).

In our animal model we investigated the biological response to different magnitude of forces in rats with similar genetic background (Alikhani et al. 2015c); therefore the results could be pooled and generalized, as it had occurred in the same individual. Further, we could predict the amount of tooth movement and saturation point based on the level of inflammatory markers expressed. If an identical magnitude of force could induce similar amount of biological response among different individuals, then from a diagnostic and treatment planning point of view, we could predict their biological response and saturation point, and in turn predict an optimal force for tooth movement on another individual. On the contrary, if an identical magnitude of force

induced different amount of biological response among different individuals, one should not extrapolate the biological response from one individual to another.

Therefore we designed this human study to address the question — does different individuals respond to an identical magnitude of force differently? Since it is unlikely to take all individual variables into account, we selected one single factor that is easy to differentiate among subjects and possible to unambiguously dichotomize them, which is the age group the subjects belong to.

The objective of this study is to investigate the expression and activity of inflammatory markers and its correlated rate of tooth movement in response to an equal magnitude of orthodontic force among different individuals, using their "age" as the variable to represent the difference among individuals.

5.3. Materials and Methods

5.3.1. Clinical Study: Subject Recruitment, Treatment Protocol and Appliance Design

A non-randomized, single-center, single-blinded clinical study was approved by the institutional review board of New York University. Healthy human subjects in both sexes and in different age ranges (age 11-14 and 21-45), regardless of their race or ethnicity, were recruited. Patients were recruited based on 1) meeting the inclusion and exclusion criteria summarized in Table 5-1, and 2) needing maxillary canine retraction of at least 3 mm. Subjects included in the study had fully erupted maxillary canines with a Class II Division 1 malocclusion that required removal of both maxillary first premolars. The overall study design was summarized in Figure 5-1.

Two orthodontic residents were trained and calibrated by the principal investigator (M.A.). They were responsible for screening and examining the subjects, determining their eligibility, and rendering the orthodontic treatment under the supervision of a faculty member who was not the principal investigator. Before starting orthodontic treatment, patients who met the selection criteria completed an informed consent form either by themselves as adults or by their guardians as minors, before starting orthodontic treatment. The subjects and the residents rendering the treatment were aware of the subjects' age and therefore not blinded. The investigators performing the measurements of samples and data analysis were blinded from the subjects' identity and age.

Routine orthodontic records were obtained from all subjects prior to orthodontic treatment, including extra/intraoral photos, panoramic radiograph, lateral cephalogram, periodontal measurements and alginate impressions. At start of orthodontic treatment, fixed

appliances were bonded on both arches (0.022” McLaughlin, Bennett, and Trevisi [MBT] prescription) including maxillary canine brackets with an auxiliary vertical slot (GAC International, Bohemia, NY, USA). Teeth were leveled and aligned with a series of sequential archwires from 0.016” NiTi, 0.016” × 0.022” NiTi, to 0.016” × 0.022” stainless steel. All subjects were monitored for oral hygiene and periodontal status in each office visit throughout the orthodontic treatment.

Patients were referred to the same surgeon for extraction of the maxillary first premolars to minimize operator variability. Canine retraction would not be initiated until leveling and aligning was achieved, and at least six months after first premolar were extracted. Periapical radiographs were taken to evaluate the morphology and integrity of canines and molars, and estimate their center of resistance based on their root length.

Canine retraction was initiated by connecting a calibrated 50-cN nickel-titanium closing coil springs (GAC International®) which generates a constant force from a power arm extending from the accessory tube of the molar bands, to a power arm extending from the ipsilateral canine bracket (Fig. 5-2). The length of the power arms was determined by the estimated location of the center of resistance using radiographs. The extended power arms allowed force application to be as close to the centers of resistance as possible, therefore facilitating bodily movement of the canines. In order to minimize the movement of adjacent teeth while canine is retracted, anterior teeth (2-2) were co-ligated as a segment with ligature wire, as well as posterior teeth from 2nd premolar to 2nd molar. The canine chosen for evaluation in each subject was randomly selected from either side to minimize the effect of uneven occlusal force due to habitual occlusion predominantly on one side.

Patients were asked to refrain from taking any pain medication, and were seen 24 hours after initiating the canine retraction for the first follow-up. At each following visit after canine retraction was initiated, the force generated by the coil was checked, and the appliances were monitored for any deformation or change in position due to chewing.

The timetable of events and data collected at different time points were summarized in Table 5-2. This clinical study was concluded after 8 weeks of canine retraction, and the subjects continued to receive orthodontic treatment at the Department of Orthodontics at New York University College of Dentistry. Routine orthodontic final records were taken at the end of treatment.

To calculate the sample size we performed a power analysis using the following formula assuming the probability of committing a type I error is 5%, and setting the power of the statistical test at 90% (power = 0.9, β = 0.1).

$$\mathrm{N} = \frac{(2\mathrm{e})\times\left(t_{\alpha,v} + t_{2(1-P),v}\right)^2}{(d)^2}$$

Where N = the sample size, e = the population standard deviation, d = the difference in means that is expected to detect (We used the results from our previous clinical study as a guide to estimate that there will be a 50% difference in cytokine expression between two age groups) (Alikhani et al. 2013), α = significance level, v = the degrees of freedom, $t_{\alpha,v}$= the t value corresponding to α and v, and P = the desired statistical power.

Based on this calculation, a sample size of 14 was suggested for this study, with 7 per group. The sample size was selected based on a type I error frequency of 5% and the power of the statistical test set at 90% (power = 0.9, β = 0.1) using our animal studies as a guide to detect at least a 50% difference in the expression of inflammatory markers.

Table 5-1. Inclusion and exclusion criteria of the clinical study	
Inclusion criteria	***Exclusion criteria***
Male and female	
Age range, 11 – 14 years or 21 – 45 years	Long-term use (6-month prior to study enrollment) of antibiotics, phenytoin, cyclosporin, anti-inflammatory drugs, systemic corticosteroids, and calcium channel blockers
Class II Division 1 malocclusion	Extreme skeletal Class II malocclusion, crossbite overjet > 10 mm, Pg-Nper > 18 mm, ANB > 7°, SN-GoGn > 38°
Have permanent dentitions at least from first molar to contralateral first molar, and need canine retraction for 3 mm or more	Primary dentitions that are not ready for comprehensive orthodontic treatments
No systemic disease	Systemic disease
No radiographic evidence of bone loss	Radiographic evidence of advanced bone loss
No history of periodontal therapy	Past periodontal disease on upper canines; past periodontal treatments during the 6-month period prior to study enrollment
Non-smokers	Current smokers
No gingivitis or untreated caries	Gingivitis and caries
No current active periodontal disease	Current periodontal disease
Probing depths < 4mm in all teeth	Probing depths > 4mm in any tooth
Good oral hygiene	Poor oral hygiene
Gingival Index ≤ 1	Gingival Index > 1
Plaque index ≤ 1	Plaque index > 1
English-speaking	Not English-speaking

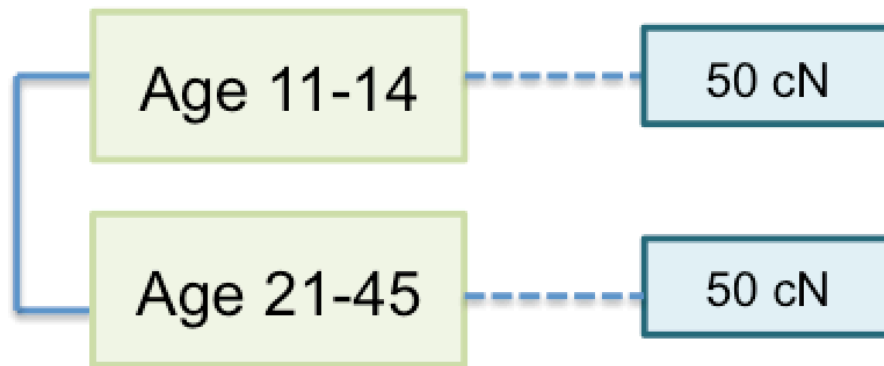
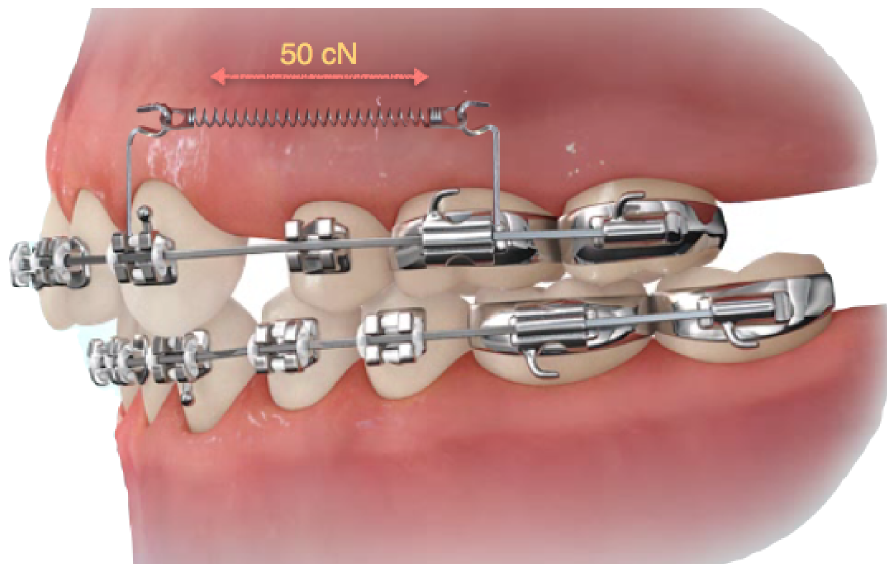


Figure 5-1. Diagram of the study design and group assignment. Subjects were healthy adolescents aged from 11 to 14, and adults aged from 21 to 25. An identical magnitude of force, 50 cN, was applied on every subject to retract canine starting at least six months after extraction of first premolar.



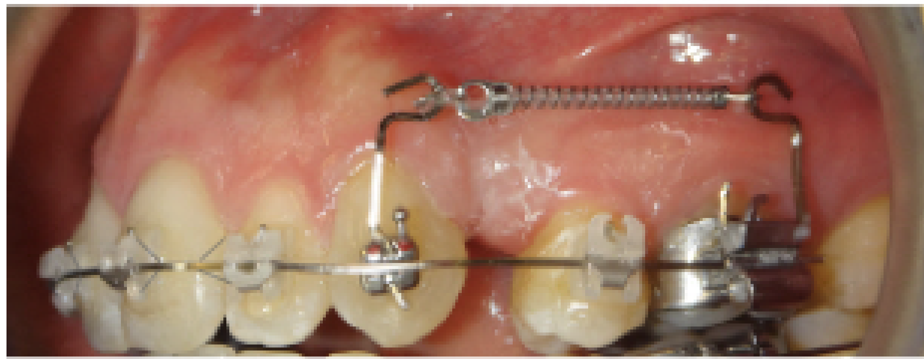


Figure 5-2. Canine retraction apparatus. Canine retraction was initiated by connecting a calibrated 50-cN nickel-titanium closing coil springs (GAC International®) which generates a constant force from a power arm extending from the accessory tube of the molar bands, to a power arm extending from the ipsilateral canine bracket. The force application was estimated to pass the centers of resistance of both canine and molar.

Table 5-2. Timetable of events during the clinical study			
Leveling and aligning to stage of .016” X .022” SS	0 – 6 months		
Placement and activation of canine retraction apparatus	≥ 6 months after extractions		
Monitoring OTM (GCF Sampling)	Pre-Tx sample (0 months)	Start of canine retraction (≥6 months)	1, 7, 14, and 28 days after canine retraction
Monitoring OH (GI, PD, PI)	Pre-Tx sample (0 months)	Start of canine retraction (≥6 months)	1, 7, 14, and 28 days after canine retraction
Intraoral photos, alginate impressions and study models	Pre-Tx sample (0 months)	Start of canine retraction (≥6 months)	28 and 56 days after canine retraction

GCF, gingival crevicular fluid; **OTM,** orthodontic tooth movement; **OH**, oral hygiene; **GI**, gingival index; **PD**, periodontal depth; **PI**, plaque index; **SS**, stainless steel; **Pre-Tx**, pre-treatment.

5.3.2. Gingival Crevicular Fluid (GCF) Sampling and Protein Analysis

To evaluate the level of inflammatory response, gingival crevicular fluid (GCF) samples were collected from the distobuccal gingival crevice of the maxillary canines (Fig. 5-3) of each subject at different time points: prior to orthodontic treatment, immediately before initiation of canine retraction, 1, 7, 14, and 28 days after the canine retraction was initiated. Collection of samples was performed between 10 AM and noon to minimize the effect of diurnal variation in GCF. If present, supra-gingival plaque was removed, and cotton rolls were used to isolate the region before GCF samples were collected with filter-paper strips (Periopaper, Oraflow Inc, Smithtown, NY, USA). One strip was carefully inserted 1 mm for 10 seconds below the gingival margin into the distobuccal gingival crevice of the canine. To avoid the contamination of GCF samples with blood, gingival index and probing depths was not assessed until GCF samples are collected.

Sample volume was assessed with Periotron 8000 (Oraflow) according to the manufacturer's instructions. Total protein amount was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA). An estimated volume of 0.6 to 1.2 μL of GCF was collected and diluted with phosphate-buffered saline (Invitrogen, Burlington, ON, Canada) to obtain 50 to 100 μL of sample required for analysis. Cytokine levels were measured using a custom glass slide-based protein array for the following cytokines: IL-1β, CCL2 (MCP1), TNF-α, RANKL, and MMP-9 (RayBiotech, Norcross, GA) according to the manufacturer's instructions

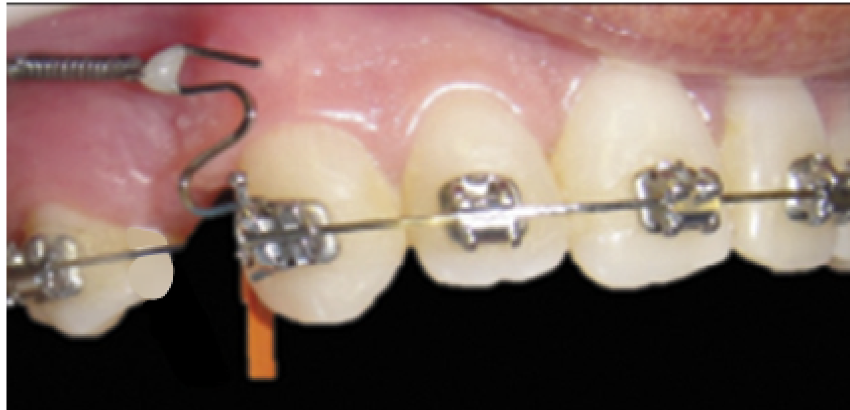


Figure 5-3. Gingival crevicular fluid (GCF) sampling. GCF was collected from the distobuccal gingival crevices of the maxillary canine of each subject at different time points to evaluate the level of inflammatory response.

5.3.3. Study Model Analysis for Rate of Tooth Movement

To evaluate the rate of canine retraction, alginate impressions were taken at the following time points: before orthodontic appliances were bonded, immediately before initiation of canine retraction, 28 days, and 56 days after canine retraction. The impressions were poured up with plaster (calcium sulfate) immediately. The models were labeled with the date taken and the patient's assigned ID number for the study. On the palatal surface of the lateral incisors and canines, vertical lines were drawn from the middle of the incisal edge to the middle of the cervical line, dividing each crown into equal halves (Fig. 5-4, A). Three landmarks along these lines were marked at the incisal edge, in the middle of the crown, and at CEJ or gingival line (Fig. 5-4, B). Distances between these landmarks on canine and its adjacent lateral incisor were measured and averaged on each model. The amount of canine retraction was calculated by comparing, i.e. subtracting the averaged distances between two selected time points, so does the rate of canine movement.

The distance between landmarks on the study models were measured using a digital caliper (Orthopli Corp, Philadelphia, PA, USA) with an accuracy of 0.01 mm. Both intra-observer and inter-observer errors were evaluated. For the evaluation of the intra-observer error, 10 models were measured twice at least 2 weeks apart. For the inter-observer error, a second investigator measured the same set of models, and the mean values of these two measurements by each investigator were compared. The random and systematic errors were calculated using a formula described by Dahlberg (Dahlberg 1940b) and Houston (Houston 1983). Both the random and systematic errors were found to be small and insignificant. Random errors were 0.031 mm for the intra-observer evaluation and 0.039 mm for the inter-observer evaluation. Systematic

errors were 0.028 mm for the intra-observer evaluation and 0.036 mm for the inter-observer evaluation ($p < 0.001$).

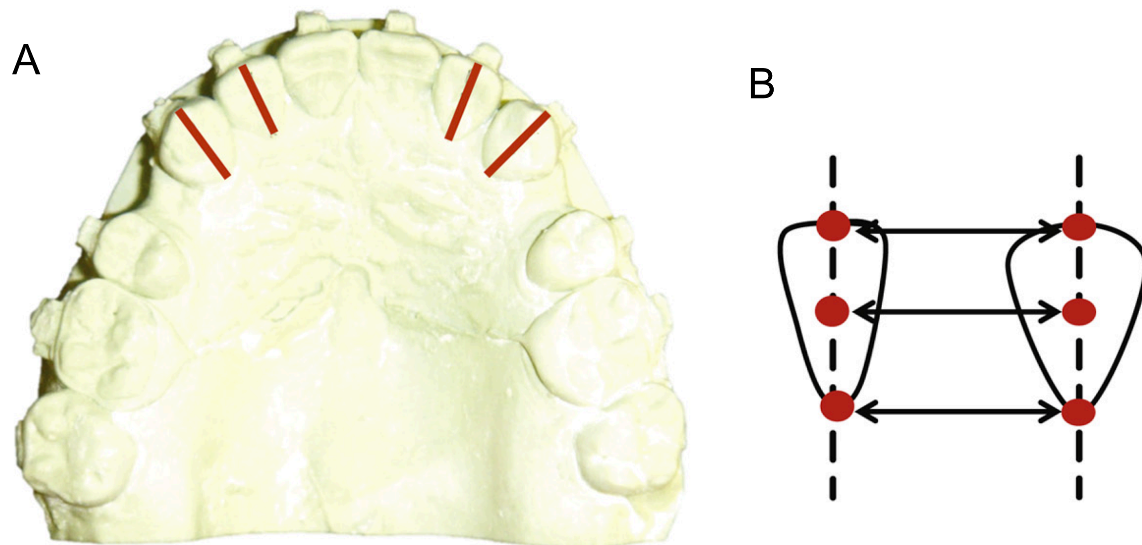


Figure 5-4. Landmarks used for analyzing rate of tooth movement on study models. Study models were obtained prior to orthodontic treatment, and 28 and 56 days after initiation of canine retraction, and the amount of tooth movement was measured between different time frames. (A) Lines that divided lateral incisors and canines into equal halves were drawn over the palatal surface of the models (*red solid lines*). (B) Three points (*red dots*) along the line were marked at the incisal edge, in the middle of the crown, and at CEJ or gingival line. Amount of tooth movement was calculated based on the measurements of the averaged distance between three landmarks on lateral incisor and canine at different time point.

5.3.4. Statistical Analysis

Comparisons between groups were assessed by analysis of variance (ANOVA). Pairwise multiple comparison analysis was performed with the Tukey's *post hoc* test. In some experiments, paired and unpaired t tests were used to compare the 2 groups. Two-tailed P values were calculated, and $p < 0.05$ was set as the level of statistical significance.

5.4. Results

5.4.1. Subject Recruitment

Fourteen subjects were recruited and completed the study with no loss to follow-up. The adolescent group (aged 11-14) comprised four females and three males, and the adult group (aged 21-45) comprised five females and two males. The subjects were recruited from patients who came to the Department of Orthodontics at New York University for comprehensive orthodontic treatment between January 2013 and December 2015. The age range of adolescent group was 12 to 14 years, with mean age of 13.3 years. The age range of adult group was 23 to 36 years, with mean age of 31 years. The patients had similar type and severities of malocclusion (Table 5-3). All patients maintained good oral hygiene throughout the study and took no additional medications, including analgesics.

Table 5-3 Comparison of the morphologic characteristics of the patients in adolescent and adult groups			
Cephalometric measurements	**Adult**	**Adolescent**	**Significance**
ANB (°)	4.3-6.1	3.9-5.2	NS
GoGn-SN (°)	25.5-32.2	27-33	NS
U1-SN (°)	104.2-111.8	103-112.2	NS
IMPA (°)	93-102	94-101	NS
Overjet (mm)	4.5-6	3.9-5.5	NS

NS, not significant ($p > 0.05$)

5.4.2. Activity of Inflammatory Markers

Both groups received similar orthodontic treatment in the leveling and aligning stage, and both groups received an identical magnitude of constant force, 50 cN, for canine retraction. GCF samples were obtained from the distobuccal gingival crevice of the canines at different time points (Table 5-2). The activities of selected inflammatory markers were measured by protein arrays, and the results were shown in Figure 5-5.

Before orthodontic treatment was initiated, protein analysis did not show significant difference in the level of concentration of IL-1β, CCL2, and TNF-α between two age groups ($p > 0.05$).

Before canine retraction was initiated (0d), the level of concentrations of IL-1β, TNF-α and CCL2 did not show significant difference between two age groups. When compared with their baseline levels before starting orthodontic treatment, none of these markers increased significantly in either of the age group ($p > 0.05$).

At 1 and 7 days after canine retraction, IL-1β increased significantly in adult group by 3.5- and 2.7- folds when compared its level of concentration immediately before canine retraction was initiated ($p < 0.05$). Afterwards, the concentration decreased, and no significant difference was observed at any later time point in comparison with the pre-retraction concentration (0d) ($p > 0.05$). In the adolescent group, IL-1β increased significantly by 1.9-fold at 1 day when compared with the pre-retraction concentration (0d) ($p < 0.05$). Afterwards, the concentration decreased and no significant difference was observed at any later time point in comparison with the pre-retraction concentration (0d) ($p > 0.05$). In addition, the level of IL-1β was significantly higher in adults than in adolescents at 1 and 7 day after retraction ($p < 0.05$),

but no significant difference between two age groups was observed at any time point after 7 days (Fig. 5-5, A).

At 1 and 7 days after canine retraction was initiated, the level of TNF-α in the adult group increased significantly by 4.2- and 2.3-fold, respectively ($p < 0.05$), when compared with the level of concentration prior to canine retraction (0d). Afterwards, the concentration decreased, and no significant difference was observed at any later time point in comparison with the pre-retraction level (0d) ($p > 0.05$). In the adolescent group, TNF-α increased significantly by 2.3-fold at 1 day when compared with the pre-retraction concentration (0d) ($p < 0.05$). Afterwards, the concentration decreased and no significant difference was observed at any later time point in comparison with the pre-retraction concentration (0d) ($p > 0.05$). The level of TNF-α was significantly higher in adults than in adolescents at 1 and 7 day after retraction ($p < 0.05$; Fig. 5-5, B).

At 1 and 7 days after canine retraction was initiated, the level of CCL2 in the adult group increased significantly by 4.3- and 2.9-fold, respectively, when compared with the level of concentration prior to canine retraction (0d). Afterwards, the concentration decreased, and no significant difference was observed at any later time point when compared with the pre-retraction level (0d) ($p > 0.05$). In the adolescent group, CCL2 increased significantly by 2.1-fold at 1 day when compared with the pre-retraction concentration (0d) ($p < 0.05$). Afterwards, the concentration decreased and no significant difference was observed at any later time point in comparison with the pre-retraction concentration (0d) ($p > 0.05$). The level of CCL2 was significantly higher in adults than in adolescents at 1 and 7 day after retraction ($p < 0.05$; Fig. 5-5, C).

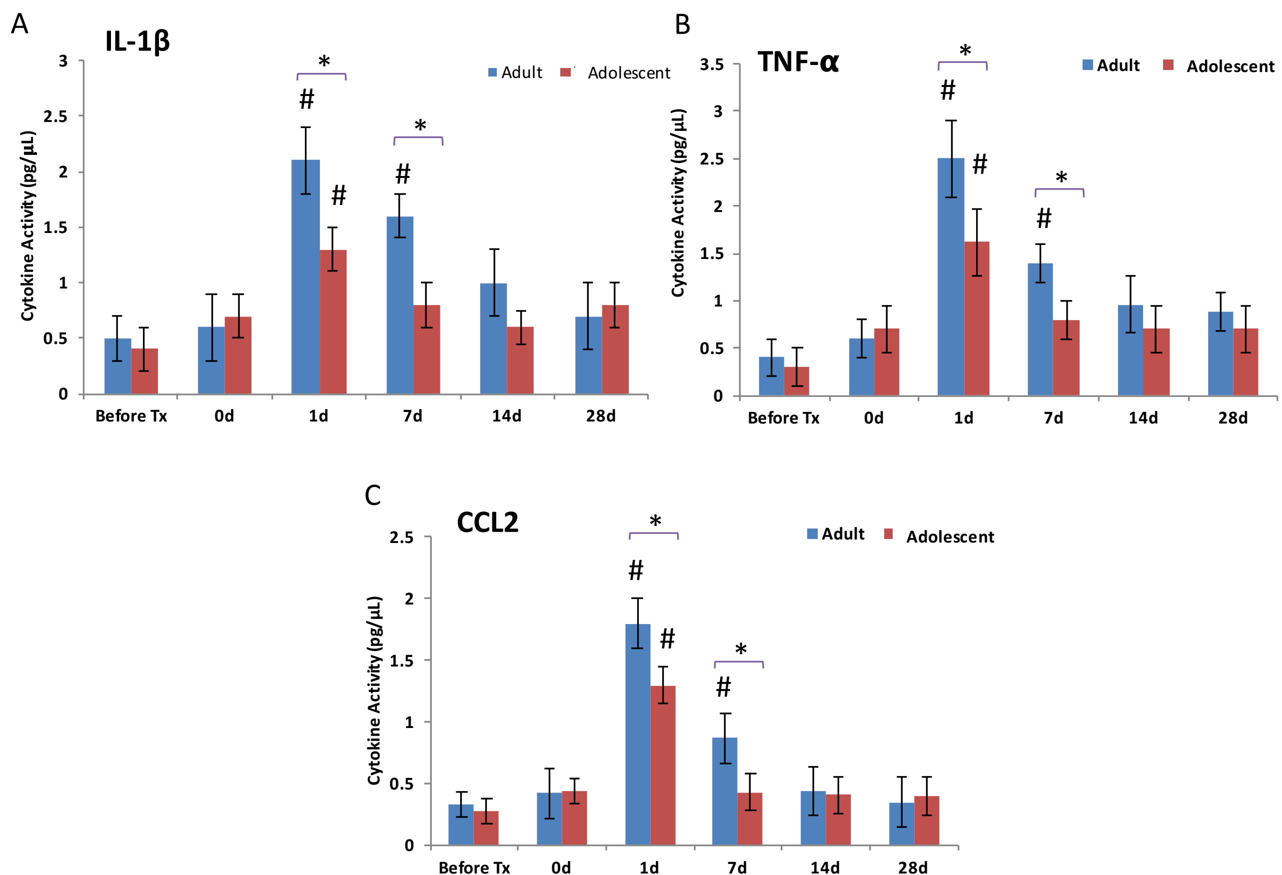


Figure 5-5. Level of cytokines and chemokines demonstrate different biological response between adolescents and adults in response to identical magnitude of force (50 cN). GCF was collected from distolabial gingival crevice of maxillary canines before starting orthodontic treatment (Before Tx), immediately before starting canine retraction (0d), and after 1, 3, 7, 14 and 28 days of activation of canine retraction apparatus. Mean concentrations of IL-1β (A), TNF-□ (B) and CCL2 (C) in both age groups were evaluated protein arrays. Each experiment was repeated 3 times, and the data was expressed as the mean ± standard deviation concentration in picograms per microliter (pg/µL) (*, significantly different between adolescent and adult group; #, significantly different from 0d within the same age group).

5.4.3. Activation of Osteoclasts

To evaluate the difference of activation of osteoclasts between two age groups in response to same amount of force, we performed protein arrays for osteoclast marker RANKL and matrix metalloproteinase 9 (MMP-9). Before canine retraction was initiated, level of RANKL and MMP-9 did not show significant increase in either of the age group ($p > 0.05$), and there was no significant difference between adolescent and adult groups (Fig. 5-6, A–B).

At one, seven, and fourteen days after canine retraction was initiated, when compared with the level of concentration immediately before canine retraction (0d), RANKL increased significantly by 2.9-, 5.8-, and 5.1-fold, respectively, in the adult group, and by 2.1-, 3.8-, and 3.7-fold, respectively, in the adolescent group ($p < 0.05$). The level of RANKL was significantly higher in adults than in adolescents ($p < 0.05$) at 7 and 14 days. At 28 days, the level of RANKL did not show significant increase in either of the age group when compared with its respective pre-retraction level, and there was no significant difference between adolescent and adult groups ($p > 0.05$; Fig. 5-6, A).

When compared with the level of pre-retraction concentration (0d) at 1, 7 and 14 days after canine retraction was initiated, MMP-9 increased significantly by 6.6-, 5.5- and 4.8-fold, respectively, in the adult group, and by 3.6-, 2.9- and 2.7-fold, respectively, in the adolescent group. Afterwards, the concentration decreased and no significant difference was observed at any later time point in comparison with the pre-retraction concentration (0d) ($p > 0.05$). The level of MMP-9 was significantly higher in adults than in adolescents at 1, 7 and 14 day after canine retraction was initiated ($p < 0.05$; Fig. 5-5, B).

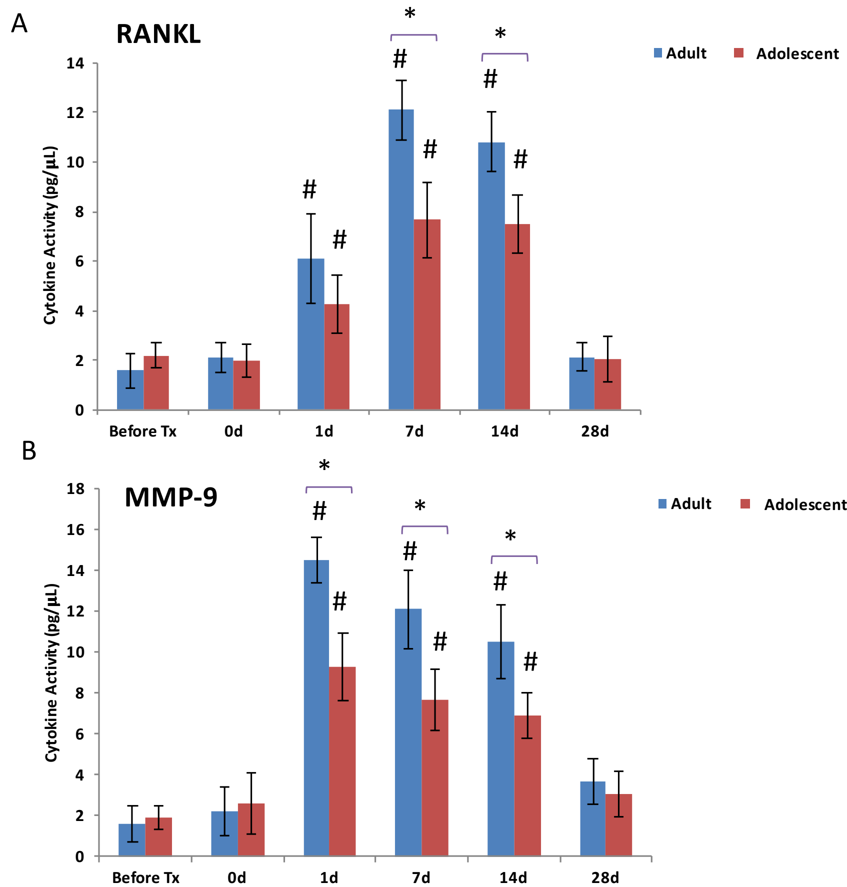


Figure 5-6. Higher level of osteoclast markers was expressed in adults in response to an identical magnitude of orthodontic force (50 cN). Mean concentration of RANKL (A) and MMP-9 (B) in the GCF collected from the distolabial gingival crevice of maxillary canines was evaluated by protein arrays at the following time points: before starting orthodontic treatment (Before Tx), immediately before starting canine retraction (0d), and 1, 3, 7, 14 and 28 days after activation of canine retraction apparatus. Each experiment was repeated 3 times, and the data was expressed as the mean ± standard deviation concentration in picograms per microliter (pg/µL) (*, significantly different between adolescent and adult group; #, significantly different from 0d within the same age group).

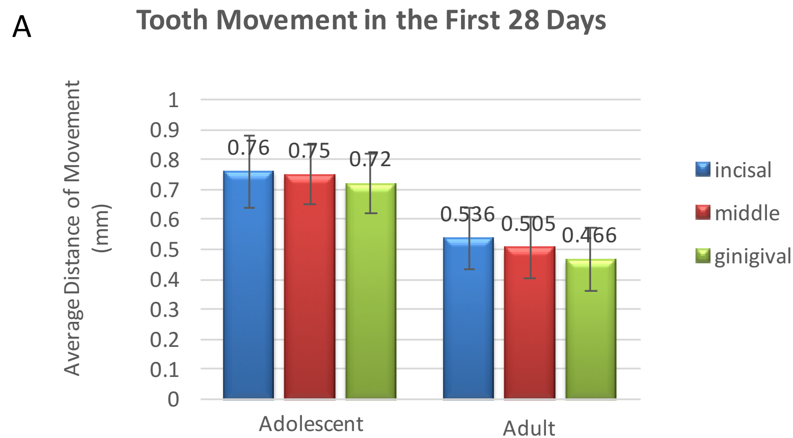
5.4.4. Rate of Tooth Movement

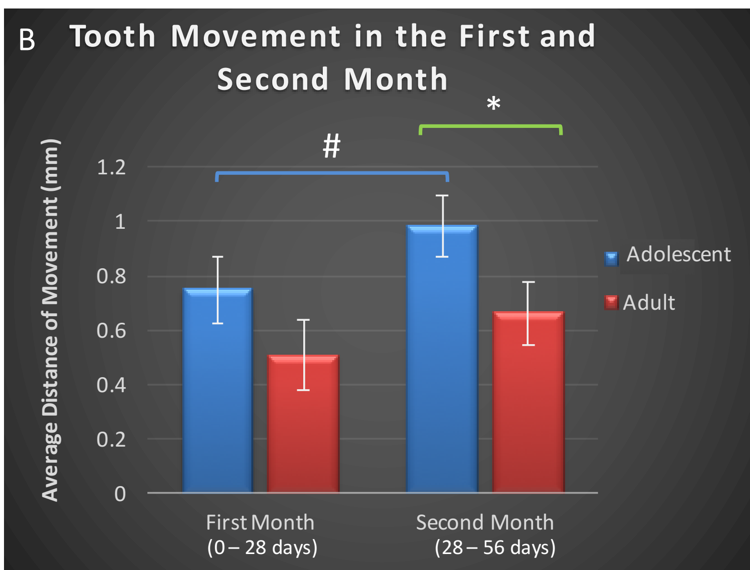
Canine retraction of different time frames was measured on the dental study models at 3 landmarks: incisal, middle, and cervical thirds of the crowns (Fig 5-4, B). The movement of the canine was almost bodily; in both the adolescent and adult groups, the incisal third of the crown moved distally slightly more than the cervical third of the crown (Fig. 5-7, A). However, this difference was not statistically significant in either of the age groups ($p < 0.05$).

During the first month of canine retraction, i.e. the first 28 days after activation of retraction appliance, the amount of canine retraction occurred in adolescents was greater than in adults (0.75 *vs.* 0.51 mm). However, the difference was not statistically significant ($p > 0.05$; Fig. 5-7, B).

During the second month of canine retraction, i.e. from 28 to 56 days after activation of retraction appliance, the amount of tooth movement was significantly greater in adolescents than in adults ($p < 0.05$). The amount of canine retraction increased in both age groups when compared with their respective amount of movement in the first month. Such increase was particularly significant in adolescents ($p < 0.05$; Fig. 5-7, B).

The total amount of canine retraction in two month was measured (Fig. 5-7, C). Significantly greater amount of canine retraction occurred in adolescents (1.56 mm in adolescents *vs.* 1.10 mm in adults; $p < 0.05$).





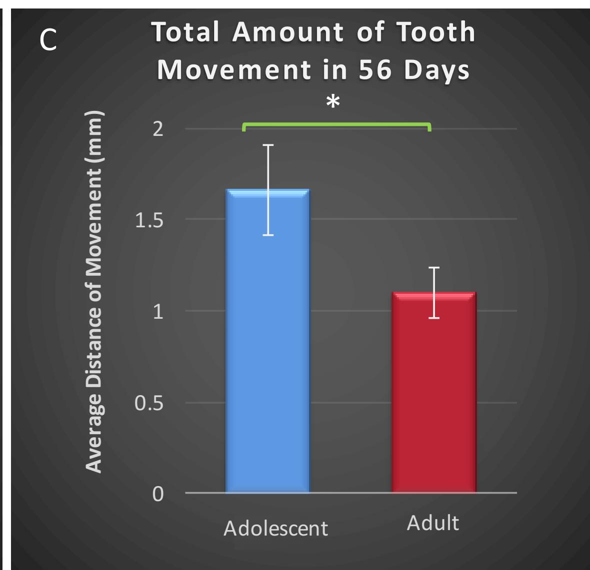


Figure 5-7. Comparison of amount of canine retraction between adolescents and adults in different time frames. (A) The graph shows the means and standard deviations of the amount of tooth movement in millimeters after 28 days at 3 landmarks (incisal, middle, and cervical thirds; Fig. 5-4) for the adolescent and adult groups. Within either of the age group, the amount of tooth movement was not significantly different among these thirds of the crown. (B) The graph shows the distance of tooth movement in the first and second month after initiation of canine retraction. In the second month (i.e. 28 to 56 days), significant faster movement was observed in

adolescents than in adults, and the amount of tooth movement is greater compared with the first month (#, significantly different within the same age group; *, significantly different between adolescent and adult groups, $p < 0.05$). (C) The graph shows the total distance of tooth movement in 56 days after initiation of canine retraction. Significant faster movement was observed in adolescents than adults. Each value represents the mean ± standard deviation movement of all subjects in their respective age group (*, significantly different between adolescent and adult groups, $p < 0.05$).

5.5. Discussion

To obtain further insights into the biological response to an identical orthodontic force among different individuals, we examined the activity of inflammatory markers and its correlated rate of tooth movement in two age groups. Many factors could affect one's biological response to orthodontic force, however, in this particular study, we chose "age" as the single variable to represent the difference among individuals, as this is a factor that is easier to dichotomize with less ambiguity. Our results demonstrated that when applying an identical magnitude of force, the adults have higher level of inflammatory response than adolescents, but slower rate of tooth movement during the first two months.

Previous studies have shown that age can play a role the rate of tooth movement. Several studies in animals (Bridges et al. 1988; Kyomen and Tanne 1997) and humans (Giannopoulou et al. 2015; Iwasaki et al. 2005; Kawasaki et al. 2006; Ren et al. 2002) with different force magnitudes, regimens, appliances and observation period have shown that juveniles tend to have greater rate of tooth movement. However, as we discussed in Chapter 4, although tooth movement is the desired result of the biological response to orthodontic forces, it may not necessarily be a precise representative of the biological response that cause tooth movement, since many other factors can affect the amount of tooth movement (Dudic et al. 2013; Krishnan and Davidovitch 2009; Ren et al. 2003a). While majority of the studies focused on evaluating the difference in the rate of tooth movement, little evidence is available in regards to the differences between different age groups in terms of their biological responses to orthodontic force, especially in human being. Previous studies comparing biological responses in GCF in different age populations lack consistency in appliance design, protocols, magnitude of force, type of tooth movement, observation period, and biomarkers evaluated (Iwasaki et al. 2005; Kawasaki et

al. 2006; Ren et al. 2002; Rody et al. 2014). Therefore this field warrants further research and the results of our present study has shed light on the difference in biological responses between juveniles and adults, by using a reliable and reproducible method including application of a constant and continuous force, and carefully selected force magnitude, age range and observation period.

The force magnitude in this clinical study was selected at 50 cN to avoid the effect of “saturation” we revealed in our animal study (Alikhani et al. 2015c), since it is most likely under saturation point in humans. If the magnitude of force had been selected at a much higher level that surpassed the saturation point of either one of the age group or both groups, then one might have observed false positive or false negative results.

The observation period of rate of movement in this study was limited to the first two months, as one-month period is a little short to observe a significant difference in human tooth movement. In addition, during the process of bone remodeling, the bone density and microenvironments surrounding the tooth constantly change over the course of tooth movement in a longer term. The difference in biological responses and rate of tooth movement under changed microenvironments within and among individuals requires further research, and is currently being investigated in our laboratory.

In this study, patients from two different age populations were recruited. We recruited patients aged 11 or older, since comprehensive fixed orthodontic treatments are usually provided to patients after most of their permanent teeth are erupted. We set age range between 21 and 45 to represent adult population in this study, since the majority of adult orthodontic patients are younger adults, according to a national survey by American Dental Association (American Dental Association 2007). The survey indicated that over 60 percent of adult orthodontic patients

are between age 20 and 39, and approximately 25 percent were between age 40 and 49. Patients older than 45 were excluded, as the biological response of older adults or elderlies is influenced by aging process, which affects various components involved in bone remodeling (Boskey and Coleman 2010; Cao et al. 2005; Chung et al. 2014; Groessner-Schreiber et al. 1991; Groessner-Schreiber et al. 1992). Besides, patients aged 15 to 20 were excluded from this study because if the biological response is different between two age groups, then by setting the lower age limit of the older group apart from the upper age limit of the younger group, the difference of biological response between two populations will be more obvious. Thus this group was excluded to decrease the possibility of getting mixed traits of subjects that may potentially confound the results.

The difference in biological response among different age groups in response to orthodontic force has been related to bone density or rate of osteoclast recruitment or activation (Bridges et al. 1988; Kyomen and Tanne 1997; Ren et al. 2005; Ren et al. 2003b). Increasing bone and mineral densities have been observed as individuals mature (Bridges et al. 1988; Burnell et al. 1980), and therefore faster movement in younger individuals has been partly attributed to lower bone and mineral densities in young bone tissue (Pilon et al. 1996; Reitan 1967). Similarly, we speculated that an increased rate of movement in the second month in both age groups is resulted from localized osteopenia caused by bone resorption during orthodontic force. Besides, while some authors argued that number of osteoclasts appears to be higher in younger rats than adult rats (Ren et al. 2005) in the early stage of orthodontic movement, others argued that the number, size and activity of osteoclasts in mechanically stressed alveolar bone during orthodontic tooth movement is the same in young and old rats (Kabasawa et al. 1996).

The presence/expression of regulatory proteins in the GCF has been recognized as a promising non-invasive diagnostic tool for monitoring orthodontic treatment outcome (Ren and Vissink 2008) since it has been shown that GCF may reflect the immune and inflammatory reactions arising from the application of orthodontic force (Kapoor et al. 2014; Ren et al. 2007; Ren et al. 2002; Uematsu et al. 1996). The inflammatory markers we selected for analysis in this study were based on their known functions, and the results from our previous studies (Alikhani et al. 2013; Teixeira et al. 2010), such as IL-1β, TNF-α, CCL2, and RANKL.

IL-1β and TNF-α are key pro-inflammatory cytokines in acute-phase inflammatory reactions, and have been implicated in the bone remodeling process during orthodontic tooth movement (Bletsa et al. 2006; Fuller et al. 2006; Garlet et al. 2007). These cytokines are produced by inflammatory cells, predominately by macrophages, and by local cells such as osteoblasts, fibroblasts, and endothelial cells. IL-1 attracts leukocytes and stimulating endothelial cells, fibroblasts, osteoclasts, and osteoblasts to enhance bone resorption and inhibit bone formation. Osteoblasts are target cells for IL-1, which in turn conveys messages to osteoclasts to resorb bone (Davidovitch 1995). TNF-α directly stimulates the differentiation of osteoclast progenitors to osteoclasts in the presence of macrophage colony-stimulating factor (M-CSF), and studies have demonstrated marked increases in in cells of the PDL and alveolar bone during orthodontic movement (Alhashimi et al. 2001; Davidovitch et al. 1988; Saito et al. 1990). Increased levels of IL-1 and TNF-a have been detected in the GCF of orthodontic patients, and they appeared to level off 24 hours after force application, suggesting a central role of these cytokines in the early phase of orthodontic tooth movement (Alhashimi et al. 2001; Grieve et al. 1994; Lee et al. 2004; Lowney et al. 1995; Uematsu et al. 1996).

RANKL has shown to be an important regulatory molecule of osteoclastogenesisis (Boyce and Xing 2007; 2008; Suda et al. 1999). It is a downstream regulator of osteoclast formation and activation. RANKL is expressed on osteoblast cell lineage and exerts its effect by binding the RANK receptor, which is expressed on osteoclasts. This binding leads to rapid differentiation of hematopoietic osteoclast precursors to mature osteoclasts. Studies have detected RANKL in osteoblasts and PDL cells during experimental tooth movement (Ogasawara et al. 2004), and significantly increased level of RANKL has been found in human GCF during orthodontic movement (Kawasaki et al. 2006; Nishijima et al. 2006; Ren and Vissink 2008). Thus, RANK and RANKL signaling as well as regulation of their expression may play critical roles in bone remodeling during orthodontic tooth movement. In addition to RANKL, in this present study we also evaluated the activation of osteoclasts by measuring the concentration of MMP-9, which is one of the osteoclast markers expressed in pre-osteoclasts and mature osteoclasts (Takeshita et al. 2000). It is one of the proteinases secreted by osteoclasts that mediates proteolysis process of bone resorption (Henriksen et al. 2011). An increased level of MMP-9 has been observed in human GCF during orthodontic movement (Capelli et al. 2011; Grant et al. 2013).

Monocyte chemoattractant/chemotatic protein-1 (MCP-1 or CCL2) plays an important role in promoting chemotaxis, differentiation, and activation of osteoclasts (Taddei et al. 2012). Upon orthodontic loading on a tooth, monocytes are recruits by CCL2 from bloodstream to the surrounding tissue to become tissue macrophages or osteoclasts. CCL2 interact with CC chemokine receptor 2 (CCR2), which is expressed by osteoclast precursors. In addition, CCL2 expression is greatly increased in periodontal tissues with orthodontic loading (Alhashimi et al.

1999; Andrade et al. 2007) in rodents and in human GCF (Alikhani et al. 2015a; Alikhani et al. 2013), as well as in other inflammatory conditions.

Our present study demonstrates that the activities of these cytokines (IL-1β, TNF-α, and RANKL) and chemokine (CCL-2) significantly elevated during orthodontic tooth movement, and responded immediately after orthodontic loading. Since all these factors play significant roles in recruitment and activation of osteoclast precursor cells, one may assume that increased activities of these factors should be accompanied by higher osteoclast activation and therefore a higher rate of tooth movement (Fuller et al. 2006; Jimi et al. 1996; O'Brien et al. 1999; Suzawa et al. 2000). However, it is partially true since adults demonstrated higher activities of above-mentioned inflammatory markers but lower rate of tooth movement, when compared with the younger individuals. Our observations implicated that there are other individual factors influencing the rate of tooth movement; therefore, one cannot predict the rate of tooth movement solely based on the level of biological responses of another individual. Rather, the level of biological response within same individual should be the basis of prediction.

Many factors other than age could affect the rate of tooth movement and warrant further research. Poor oral hygiene, periodontal disease, advanced alveolar bone loss, systemic diseases, and anti-inflammatory medications can affect the rate of tooth movement significantly (Bartzela et al. 2009; Knop et al. 2012; Okamoto et al. 2009). To minimize the influence from these factors, we set clear exclusion criteria, as summarized in Table 5-1, and the subjects were able to maintain good oral hygiene.

It has been shown that the forces of occlusion can affect the rate of tooth movement significantly (Usumi-Fujita et al. 2013). To rule out the effect of occlusion in this study, we selected patients with similar type and severities of malocclusion (Table 5-3). Patients with

crossbite or deviation during closure caused by occlusal interference were not included in this study. In addition, to minimize the possibility of uneven occlusal forces due to habitual occlusion predominantly on one side, the canine chosen for data collection in each subject was randomly selected from either side. Furthermore, the rationale of studying biological response and rate of tooth movement by using canine retraction model is that in patients with Class II Division 1 occlusion, the canines are usually free from occlusal interferences. During canine retraction in this study, occlusal interference was carefully checked, but no subject needed occlusal adjustment.

Type of tooth movement, such as tipping *versus* bodily movement, can affect the rate of orthodontic tooth movement (Lee 1995; Shpack et al. 2008). In this study, we designed the canine retraction apparatus (Fig. 5-2) in a particular way to facilitate bodily movement and minimize uncontrolled tipping. Although our results suggested that canine retraction was not completely bodily, and some degree of tipping movement was observed in both age groups, the tilting was not significant within and between the age groups (Fig. 5-7, A). Therefore, tipping by itself cannot fully explain the difference in the rates of tooth movement observed between adults and adolescents.

Levels of sex hormones in women throughout the estrous cycle could be another confounding variable that could affect the rate of bone remodeling and tooth movement (Haruyama et al. 2002; Zittermann et al. 2000). Unfortunately, we could not control this variable by recruiting subjects of same sex due to somewhat limited number of candidates for this study.

Extractions can accelerate the rate of tooth movement by significantly increasing the activity of inflammatory markers, which could affect the results of our study. Therefore, extraction was performed at least six months before initiation of canine retraction.

The canine retraction in the present study was achieved by using nickel-titanium closing coil springs, which are able to provide a constant force during the study without the need for re-activation. The load deflection analysis for the 50-cN spring showed that the force level remained relatively constant for decreases of 0.5 to 1.5 mm in the length of the spring after initial activation (data not shown).

No patient in this clinical study showed any evidence of significant root resorption or advanced alveolar bone loss in the routine panoramic radiographs taken as final record. However, panoramic or periapical radiographs are not precise for measuring the magnitude of root resorption, and future studies are necessary (Dudic et al. 2009; Dudic et al. 2008; Sameshima and Asgarifar 2001).

While the result of our animal study suggests using the level of the inflammatory response as a predicative factor for the rate of tooth movement, the result from this clinical study indicated that doing so would be erroneous without considering individual variables such as age. Therefore, one should always compare the level of inflammatory markers within the same individual.

5.6. Conclusions

1. Individual variability can affect biological response to orthodontic force, therefore patients can react differently to an identical magnitude of orthodontic force.
2. Individuals of different ages demonstrate different levels of inflammatory markers to an identical magnitude of force. The adults have higher level of inflammatory markers and osteoclast activation than adolescents; however, adults have lower rate of tooth movement during the study period.
3. The level of inflammatory markers among different individuals cannot be directly used for comparison to predict rate of tooth movement. Instead, one should compare the level of biological response within the same individual.

Chapter 6. Saturation of biological response and rate of tooth movement among individuals: A clinical study

This Chapter focused on research strategy and results to our ***Specific Aim 3***, which is to investigate the activity of inflammatory markers and the correlated effect on the rate of orthodontic tooth movement among individuals of different ages while using different force magnitudes.

- Sub-aim 3A: To investigate the inflammatory marker activity in response to different orthodontic force magnitudes in different age groups.
- Sub-aim 3B: To investigate the rate of tooth movement in response to different orthodontic force magnitudes in different age groups.

6.1. Abstract

Objectives: Investigate the activity of inflammatory markers and the correlated effect on the rate of orthodontic tooth movement in response to different force magnitudes in two age groups.

Methods and Materials: Healthy human subjects in both sexes and in different age groups (age 11-14 and 21-45) were recruited. Each subject in both age groups was randomly assigned to receive one of the following four magnitudes of constant force for canine retraction: 50, 100, 150 and 200 cN. Gingival crevicular fluid (GCF) from distolabial crevice was collected at one day after retraction. The activity of different inflammatory markers in GCF including IL-1β, CCL2, and RANKL were measured using antibody-based assays. The rate of canine retraction in 28 days was measured on study models. Differences within and between groups were assessed.

Results: 32 subjects were recruited with 4 subjects in each force magnitude subgroup and 16 per age group. There was a linear relation between the force and the level of cytokine activities during lower magnitudes of force. However, higher magnitudes of force did not increase the cytokine activities in either age group, i.e. activity of cytokines reached a saturation point. Adolescents have lower cytokine activities at lower magnitudes of force than adults, but the activities increased at higher force magnitudes while adults have reached saturation. The rate of canine movement in 28 days was not significantly different between adolescents and adults, however, higher magnitude of force did not increase the rate of movement, and the rate tended to be faster in adolescents in all force groups.

Conclusion: Saturation of biological response exists in humans. After reaching certain magnitude of force, biological response is saturated, and higher magnitude of force does not increase inflammatory markers nor amount of tooth movement. Adolescents has higher saturation point than adults.

6.2. Introduction

In our animal study depicted in Chapter 4, we have demonstrated that increase of force magnitude is accompanied with higher levels of cytokine and chemokine expression only up to a certain point (Alikhani et al. 2015b; Alikhani et al. 2015c). Increasing the magnitude of force beyond that point did not produce higher levels of inflammatory markers, osteoclast formation, nor were higher rate of tooth movement achieved. This observation led to the conclusion that there is a "biological saturation point" that a higher magnitude of force beyond which saturation point is reached, will not induce higher amount of biological response. According to this theory, at a lower range of force magnitude, there is a linear relationship between the magnitude of force and the level of inflammation; after reaching a certain magnitude of force, the area of necrotic zone (i.e. hyalinization; cell-free zone) in the PDL is maximized, and further inflammation is not possible until the necrotic tissues are cleared by remodeling mechanism. This can explain the reason why the application of a higher force does not necessarily produce more tooth movement, and therefore the saturation of the biological response must be overcome by other methods instead of applying heavier force.

In our clinical study depicted in Chapter 5, we demonstrated that there are differences in biological response and rate of tooth movement to an identical orthodontic force among different individuals. We chose "age" as the single variable to differentiate different individuals, and demonstrated that when applying an identical magnitude of force, the adults have higher level of inflammatory response than adolescents, but slower rate of tooth movement during the first two months.

While the result of our animal study suggests using the level of the inflammatory response as a predicative factor for the rate of tooth movement, the result from our clinical study

in Chapter 5 indicated that it would be erroneous without considering individual variables such as age. Therefore, one should always compare the level of inflammatory markers within the same individual, and should not extrapolate a result from one person to another.

To date, saturation in biological response to orthodontic force has never been demonstrated or proved in humans. Assuming such saturation phenomenon exists in humans, the next question would be whether saturation points are identical or different among individuals. Since we have proved that different biological responses exist among different individuals in response to the same orthodontic stimuli, it is logical to speculate that saturation point is different among different individuals. Current literature has no information in this regard; therefore, to address these questions, we designed the following human study. If saturation point was the same among individuals, then one could predict saturation point and the optimal force for tooth movement in another individual. On the contrary, if saturation point was different among individuals, then different amount of magnitude of force should be considered in different individuals to optimize the rate of tooth movement.

For the same rationale as our previous clinical study that we explained in Chapter 5, one single factor, "age," served as the representative differentiating factor in our study. Since it is unlikely to take all individual variables into account, we selected one single factor that is easy to differentiate among subjects and possible to unambiguously dichotomize them, which is the age group the subjects belong to.

The objectives of the study are to investigate the existence of biological saturation to orthodontic force in humans, and to investigate the difference in saturation point among different individuals.

6.3. Materials and Methods

6.3.1. Clinical Study: Subject Recruitment, Treatment Protocol, and Appliance Design

A non-randomized, single-center, single-blinded clinical study was approved by the institutional review board of New York University. Healthy human subjects in both sexes and in different age ranges (age 11-14 and 21-45), regardless of their race or ethnicity, were recruited. Patients were recruited based on 1) meeting the inclusion and exclusion criteria summarized in Table 6-1, and 2) needing maxillary canine retraction of at least 3 mm. Subjects included in the study had fully erupted maxillary canines with a Class II Division 1 malocclusion that required removal of both maxillary first premolars. The overall study design was summarized in Figure 6-1.

Two orthodontic residents were trained and calibrated by the principal investigator (M.A.) They were responsible for screening and examining the subjects, determining their eligibility, and rendering the orthodontic treatment under the supervision of a faculty member who was not the principal investigator. Before starting orthodontic treatment, patients who met the selection criteria completed an informed consent form either by themselves as adults or by their guardians as minors, before starting orthodontic treatment. The subjects and the residents rendering the treatment were aware of the subjects' age and therefore not blinded. The investigators performing the measurements of samples and data analysis were blinded from the subjects' identity and age.

Routine initial orthodontic records were obtained from all subjects prior to orthodontic treatment, including extra/intraoral photos, panoramic radiograph, lateral cephalogram, periodontal measurements and alginate impressions. At start of orthodontic treatment, fixed

appliances were bonded on both arches (0.022" McLaughlin, Bennett, and Trevisi [MBT] prescription) including maxillary canine brackets with an auxiliary vertical slot (GAC International, Bohemia, NY, USA). Teeth were leveled and aligned with a series of sequential archwires from 0.016" NiTi, 0.016" × 0.022" NiTi, to 0.016" × 0.022" stainless steel. All subjects were monitored for oral hygiene and periodontal status in each office visit throughout the orthodontic treatment.

Patients were referred to the same surgeon for extraction of the maxillary first premolars to minimize operator variability. Canine retraction would not be initiated until leveling and aligning was achieved, and at least six months after first premolar were extracted. Periapical radiographs were taken to evaluate the morphology and integrity of canines and molars, and estimate their center of resistance based on their root length.

Canine retraction was initiated by applying a calibrated nickel-titanium closing coil springs (GAC International®) which generates a constant force at a designated magnitude of force. Each subject in both age groups was randomly assigned to receive one of the following four magnitudes of force: 50, 100, 150 and 200 cN. The coil spring was connected from a power arm extending from the accessory tube of the molar bands, to a power arm extending from the ipsilateral canine bracket (Fig. 6-2). The length of the power arms was determined by the estimated location of the center of resistance using radiographs. The extended power arms allowed force application to be as close to the centers of resistance as possible, therefore facilitating bodily movement of the canines. In order to minimize the movement of adjacent teeth while canine is retracted, anterior teeth (2-2) were co-ligated as a segment with ligature wire, as well as posterior teeth from 2nd premolar to 2nd molar. The canine chosen for evaluation in each

subject was randomly selected from either side to minimize the effect of uneven occlusal force due to habitual occlusion predominantly on one side.

Patients were asked to refrain from taking any pain medication, and were seen 24 hours after initiating the canine retraction for the first follow-up. At each following visit after canine retraction was initiated, the force generated by the coil was checked, and the appliances were monitored for any deformation or change in position due to chewing.

The timetable of events and data collected at different time points were summarized in Table 6-2. This clinical study was concluded after 4 weeks of canine retraction, and the subjects continued to receive orthodontic treatment at the Department of Orthodontics at New York University College of Dentistry. Routine orthodontic final records were taken at the end of treatment.

To calculate the sample size we performed a power analysis using the following formula assuming the probability of committing a type I error is 5%, and setting the power of the statistical test at 90% (power = 0.9, β = 0.1).

$$\mathrm{N} = \frac{(2\mathrm{e})\times\left(t_{\alpha,v} + t_{2(1-P),v}\right)^2}{(d)^2}$$

Where N = the sample size, e = the population standard deviation, d = the difference in means that is expected to detect (We used the results from our previous clinical study (Alikhani et al. 2013) as a guide to estimate that there will be a 50% difference in cytokine expression between two age groups), α = significance level, v = the degrees of freedom, $t_{\alpha,v}$= the t value corresponding to α and v, and P = the desired statistical power.

Based on this calculation, a sample size of 32 was suggested for this study, with 4 per force magnitude group, and 16 per age group. The sample size was selected based on a type I

error frequency of 5% and the power of the statistical test set at 90% (power = 0.9, $\beta = 0.1$) using our animal studies as a guide to detect at least a 50% difference in the expression of inflammatory markers.

Table 6-1. Inclusion and exclusion criteria of the clinical study	
Inclusion criteria	***Exclusion criteria***
Male and female	
Age range, 11 – 14 years or 21 – 45 years	Long-term use (6-month prior to study enrollment) of antibiotics, phenytoin, cyclosporin, anti-inflammatory drugs, systemic corticosteroids, and calcium channel blockers
Class II Division 1 malocclusion	Extreme skeletal Class II malocclusion, crossbite overjet > 10 mm, Pg-Nper > 18 mm, ANB > 7°, SN-GoGn > 38°
Have permanent dentitions at least from first molar to contralateral first molar, and need canine retraction for 3 mm or more	Primary dentitions that are not ready for comprehensive orthodontic treatments
No systemic disease	Systemic disease
No radiographic evidence of bone loss	Radiographic evidence of advanced bone loss
No history of periodontal therapy	Past periodontal disease on upper canines; past periodontal treatments during the 6-month period prior to study enrollment
Non-smokers	Current smokers
No gingivitis or untreated caries	Gingivitis and caries
No current active periodontal disease	Current periodontal disease
Probing depths < 4mm in all teeth	Probing depths > 4mm in any tooth
Good oral hygiene	Poor oral hygiene
Gingival Index ≤ 1	Gingival Index > 1
Plaque index ≤ 1	Plaque index > 1
English-speaking	Not English-speaking

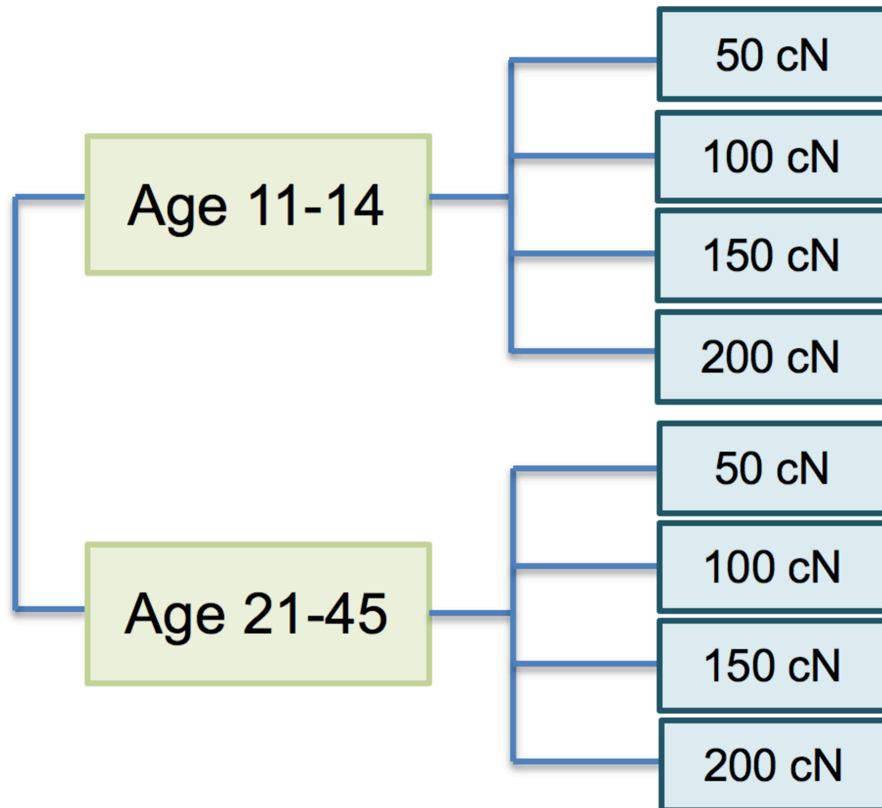


Figure 6-1. Diagram of the study design and group assignment. Subjects were healthy adolescents aged from 11 to 14, and adults aged from 21 to 25. One of four magnitudes of force (50, 100, 150 or 200 cN) was randomly assigned to each subject in both age groups to retract canine. Canine retraction was started at least six months after extraction of first premolar.

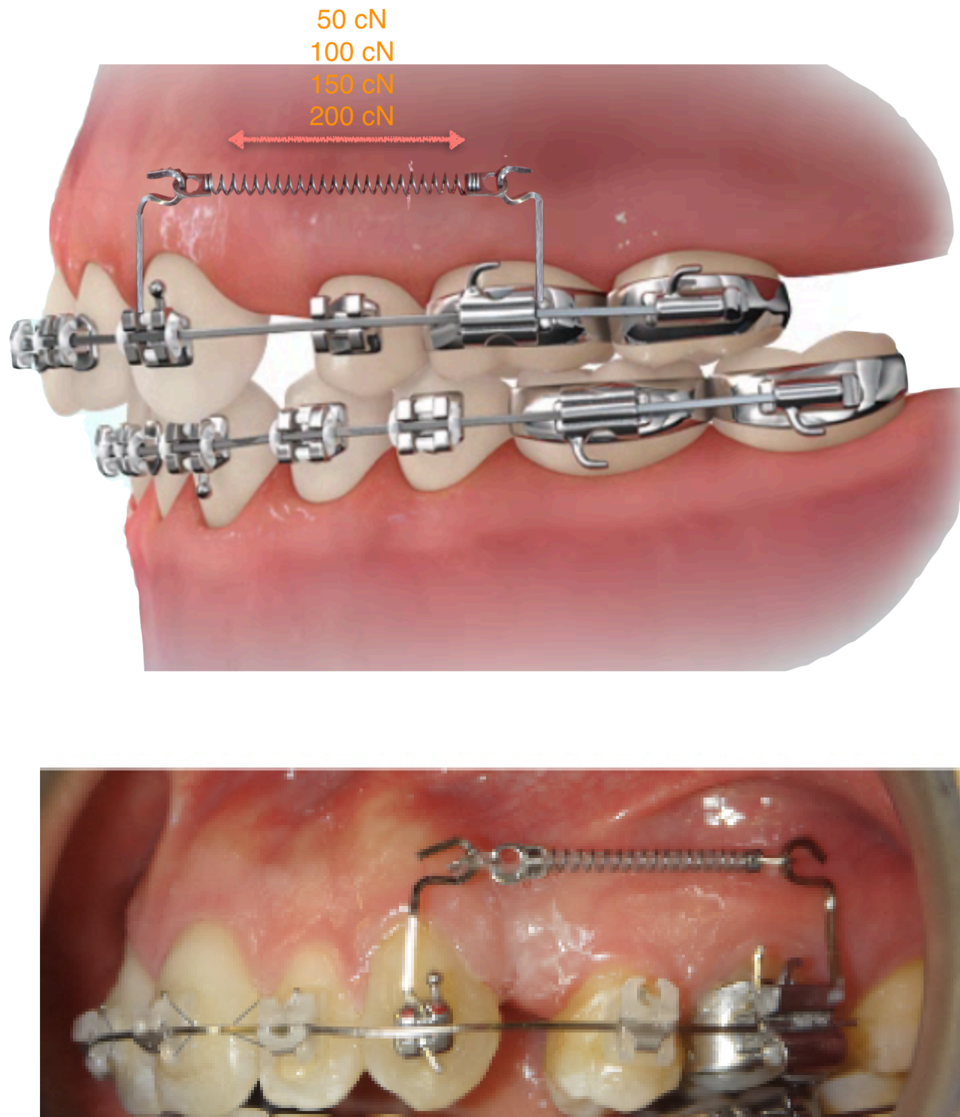


Figure 6-2. *Canine retraction apparatus*. Canine retraction was initiated by connecting a calibrated nickel-titanium closing coil springs (GAC International®) which generates a constant force at a selected magnitude from a power arm extending from the accessory tube of the molar bands, to a power arm extending from the ipsilateral canine bracket. The force application was estimated to pass the centers of resistance of both canine and molar.

Table 6-2. Timetable of events during the clinical study			
Leveling and aligning to stage of .016” X .022” SS	0 – 6 months		
Placement and activation of canine retraction apparatus	≥ 6 months after extractions		
Monitoring OTM (GCF Sampling)			1 day after canine retraction
Monitoring OH (GI, PD, PI)	Pre-Tx sample (0 months)	Start of canine retraction (≥6 months)	1 and 28 days after canine retraction
Intraoral photos, alginate impressions and study models	Pre-Tx sample (0 months)	Start of canine retraction (≥6 months)	28 days after canine retraction

GCF, gingival crevicular fluid; **OTM,** orthodontic tooth movement; **OH**, oral hygiene; **GI**, gingival index; **PD**, periodontal depth; **PI**, plaque index; **SS**, stainless steel; **Pre-Tx**, pre-treatment.

6.3.2. Gingival Crevicular Fluid (GCF) Sampling and Protein Analysis

To evaluate the level of inflammatory response, gingival crevicular fluid (GCF) samples were collected from the distobuccal gingival crevice of the maxillary canines of each subject one day after retraction was initiated (Fig. 6-3). Collection of samples was performed between 10 AM and noon to minimize the influence from diurnal variation. If present, supra-gingival plaque was removed, and cotton rolls were used to isolate the region before GCF samples were collected with filter-paper strips (Periopaper, Oraflow Inc, Smithtown, NY, USA). One strip was carefully inserted 1 mm for 10 seconds below the gingival margin into the distobuccal gingival crevice of the canine. To avoid the contamination of GCF samples with blood, gingival index and probing depths was not assessed until GCF samples are collected.

Sample volume was assessed with Periotron 8000 (Oraflow) according to the manufacturer's instructions. Total protein amount was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA). An estimated volume of 0.6 to 1.2 μL of GCF was collected and diluted with phosphate-buffered saline (Invitrogen, Burlington, ON, Canada) to obtain 50 to 100 μL of sample required for analysis. Cytokine levels were measured using a custom glass slide-based protein array for the following cytokines: IL-1β, CCL2 (MCP1), and RANKL (RayBiotech, Norcross, GA) according to the manufacturer's instructions.

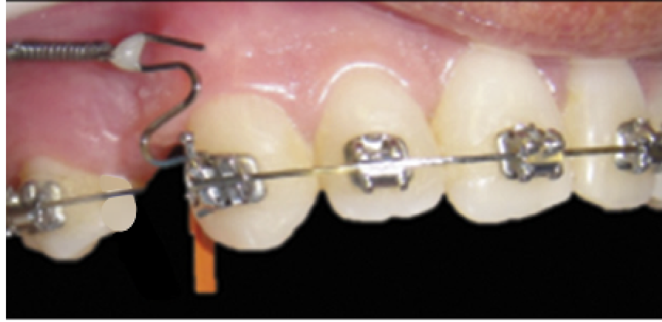


Figure 6-3. Gingival crevicular fluid (GCF) was collected from the distobuccal gingival crevices of the maxillary canine of each subject at one day after canine retraction to evaluate the level of inflammatory response.

6.3.3. Study Model Analysis for Rate of Tooth Movement

To evaluate the rate of canine retraction, alginate impressions were taken at the following time points: before orthodontic appliances were bonded, immediately before initiation of canine retraction, and 28 days after canine retraction. The impressions were poured up with plaster (calcium sulfate) immediately. The models were labeled with the date taken and the patient's assigned ID number for the study. On the palatal surface of the lateral incisors and canines, vertical lines were drawn from the middle of the incisal edge to the middle of the cervical line, dividing each crown into equal halves (Fig. 6-4, A). Three landmarks along these lines were marked at the incisal edge, in the middle of the crown, and at CEJ or gingival line (Fig. 6-4, B). Distances between these landmarks on canine and its adjacent lateral incisor were measured and averaged on each model. The amount of canine retraction was calculated by comparing, i.e. subtracting the averaged distances before and 28 days after canine retraction.

The distance between landmarks on the study models were measured using a digital caliper (Orthopli Corp, Philadelphia, PA, USA) with an accuracy of 0.01 mm. Both intra-observer and inter-observer errors were evaluated. For the evaluation of the intra-observer error, 10 models were measured twice at least 2 weeks apart. For the inter-observer error, a second investigator (M.A.) measured the same set of models twice, and the mean values of these two measurements by each investigator were compared. The random and systematic errors were calculated using a formula described by Dahlberg (Dahlberg 1940b) and Houston (Houston 1983). Both the random and systematic errors were found to be small and insignificant. Random errors were 0.031 mm for the intra-observer evaluation and 0.039 mm for the inter-observer evaluation. Systematic errors were 0.028 mm for the intra-observer evaluation and 0.036 mm for the inter-observer evaluation ($p < 0.001$).

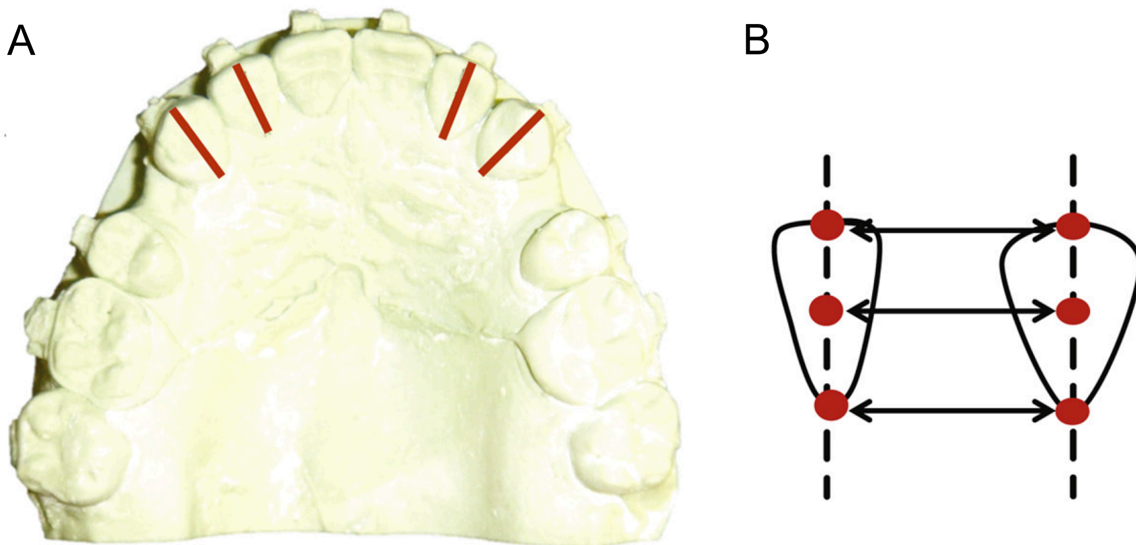


Figure 6-4. Landmarks used for analyzing rate of tooth movement on study models. Study models were obtained prior to orthodontic treatment, immediately before and 28 days after initiation of canine retraction. The amount of tooth movement was measured between before and after canine retraction. (A) Lines that divided lateral incisors and canines into equal halves were drawn over the palatal surface of the models (*red solid lines*). (B) Three points (*red dots*) along the line were marked at the incisal edge, in the middle of the crown, and at CEJ or gingival line. Amount of tooth movement was calculated based on the measurements of the averaged distance between three landmarks on lateral incisor and canine.

6.3.4. Statistical Analysis

Comparisons between groups were assessed by analysis of variance (ANOVA). Pairwise multiple comparison analysis was performed with the Tukey's *post hoc* test. In some experiments, paired and unpaired t tests were used to compare the 2 groups. Two-tailed p values were calculated, and $p < 0.05$ was set as the level of statistical significance.

6.4. Results

6.4.1. Subject Recruitment

Thirty-two subjects were recruited and completed the study with no loss to follow-up. Four subjects were in each force magnitude subgroup and sixteen per age group. The adolescent group (aged 11-14) comprised 11 females and 5 males, and the adult group (aged 21-45) comprised 9 females and 7 males. The subjects were recruited from patients who came to the Department of Orthodontics at New York University for comprehensive orthodontic treatment between January 2013 and December 2015. The age range of adolescent group was 12 to 14 years, with mean age of 12.8 years. The age range of adult group was 26 to 42 years, with mean age of 35.4 years. The patients had similar type and severities of malocclusion (Table 6-3). All patients maintained good oral hygiene throughout the study and took no additional medications, including analgesics.

Table 6-3 Comparison of the morphologic characteristics of the patients in adolescent and adult groups			
Cephalometric measurements	**Adult**	**Adolescent**	**Significance**
ANB (°)	3.9-5.9	4-6	NS
GoGn-SN (°)	25.4-32	27-33	NS
U1-SN (°)	103-109	102-112.5	NS
IMPA (°)	93-102	95-103	NS
Overjet (mm)	4.5-6	4-6.5	NS

NS, not significant ($p > 0.05$)

6.4.2. Activity of Inflammatory Markers

All subjects received similar orthodontic treatment in the leveling and aligning stage. Each subject was randomly assigned to receive a specific magnitude of force for canine retraction, ranging from 50 to 200 cN. GCF samples were collected from the distobuccal gingival crevice of the canines one day after activation of the retraction apparatus (Table 6-2). The activities of selected inflammatory markers were measured by protein arrays, and the results were shown in Figure 6-5.

6.4.2.1. Interleukin-1β (Fig. 6-5, A)

In the adult group, when compared with 50 cN, the level of IL-1β increased significantly in 100, 150 and 200 cN groups by 1.4-, 1.5-, and 1.4-fold, respectively ($p < 0.05$). However, the difference in the level of concentration of IL-1β was not significant between 100 and 150 cN, between 100 and 200 cN, or between 150 and 200 cN groups ($p > 0.05$).

In the adolescent group, when compared with 50 cN, the level of IL-1β increased significantly in 100, 150 and 200 cN groups by 1.8-, 2.8-, and 2.7-fold, respectively ($p < 0.05$). When compared with 100 cN, the level of IL-1β increased significantly in 150 and 200 cN groups by 1.55-, and 1.45-fold, respectively ($p < 0.05$). However, the difference in the level of concentration was not significant between 150 and 200 cN groups ($p > 0.05$).

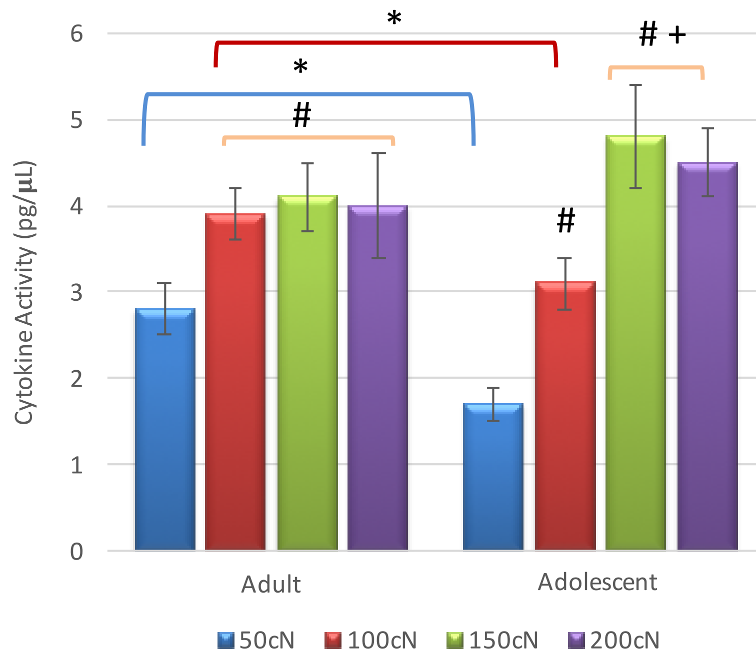
In terms of comparison between two groups, the levels IL-1β were significantly higher in adults who received 50 and 100 cN force, when compared with the adolescents who received the same magnitude of force ($p < 0.05$).

6.4.2.2. Monocyte Chemoattractant Protein-1 (MCP-1 / CCL2) (Fig. 6-5, B)

In the adult group, in comparison with 50 cN, the level of CCL2 increased significantly in 100, 150 and 200 cN groups by 1.73-, 1.77-, and 1.68-fold, respectively ($p < 0.05$). However, the difference in the level of concentration was not significant between 100 and 150 cN groups, or 100- and 200-cN group ($p > 0.05$).

In the adolescent group, in comparison with 50 cN, the level of CCL2 increased significantly in 100-, 150- and 200-cN groups by 2.1-, 3.6-, and 4.0-fold, respectively ($p < 0.05$). When compared with 100 cN, the level of CCL2 increased significantly in 150- and 200-cN groups by 1.7-, and 1.9-fold, respectively ($p < 0.05$). However, the difference in the level of concentration was not significant between 150- and 200-cN group ($p > 0.05$). The level of CCL2 was significantly higher in adults who received 50- and 100-cN force, when compared with the adolescents who received the same magnitude of force. On the contrary, the level of CCL2 was significantly higher in adolescents who received 150- and 200-cN force, when compared with the adults who received the same magnitude of force ($p < 0.05$)

The results has demonstrated that in adult group, there is an initial increase in the activity of inflammatory cytokines when forces increased from 50 to 100 cN, and then a plateau from 100 to 200-cN force levels; meanwhile, in the adolescent group, an initial increase in cytokine activities occurred when forces increased from 50 to 150 cN, and then a plateau from 150 to 200-cN force levels.


A. IL-1β
Cytokine Activity (pg/µL)
6
5
4
3
2
1
0
*
*
#
+
#
Adult
Adolescent
50cN
100cN
150cN
200cN

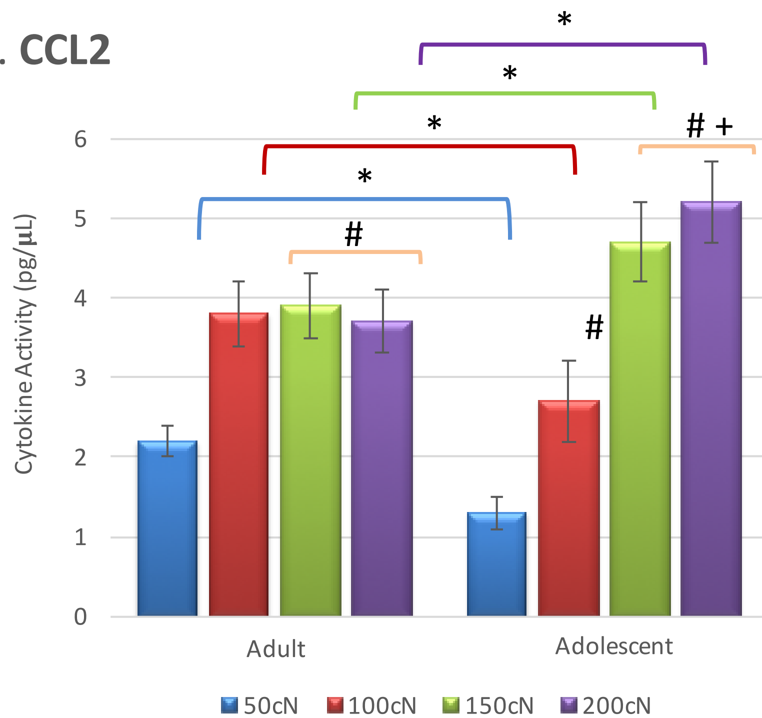

B. CCL2
Cytokine Activity (pg/µL)
6
5
4
3
2
1
0
*
*
*
*
#
+
#
Adult
Adolescent
50cN
100cN
150cN
200cN

Figure 6-5. Activity of IL-1β and CCL2 demonstrate saturation in response to higher magnitude of force, and different saturation point between adolescents and adults. GCF was collected from distolabial gingival crevice of maxillary canines one day after activation of canine retraction apparatus. Mean concentrations of IL-1β (A) and CCL2 (B) in both age groups were evaluated by protein arrays. Each experiment was repeated 3 times, and the data was expressed as the mean ± standard deviation concentration in picograms per microliter (pg/μL) (#, significantly different from 50-cN group within the same age group; +, significantly different from 100-cN group within the same age group; *, significantly different between adolescents and adults who received the same force magnitude).

6.4.3. Activation of Osteoclasts

To evaluate the effect of different magnitude of force on activation of osteoclasts between two age groups, samples were collected from the distobuccal gingival crevice of the canines one day after activation of the retraction apparatus. The activities of osteoclast marker RANKL was measured by protein arrays, and the results were shown in Figure 6-6.

In the adult group, when compared with 50-cN group, the level of RANKL increased significantly in 100, 150 and 200-cN groups by 1.55-, 1.52-, and 1.3-fold, respectively ($p < 0.05$; Fig. 6-6). However, the difference in the level of concentration was not significant between 100- and 150-cN groups, nor between 100- and 200-cN groups ($p > 0.05$).

In the adolescent group, when compared with 50 cN, the level of RANKL increased significantly in 100-, 150- and 200-cN group by 1.4-, 2.1-, and 2.3-fold, respectively ($p < 0.05$). When compared with 100 cN, the level of RANKL increased significantly in 150- and 200-cN groups by 1.5-, and 1.6-fold, respectively ($p < 0.05$). However, no significant differences were observed between adolescents who received 150 and 200-cN force ($p > 0.05$). The level of RANKL was significantly higher in adults who received 100-cN force, when compared with the adolescents who received the same magnitude of force. On the contrary, the level of RANKL was significantly higher in adolescents who received 200-cN force when compared with the adults who received the same magnitude of force ($p < 0.05$).

The results has demonstrated that in adult group, there is an initial increase in the activity of osteoclast marker when forces increased from 50- to 100-cN, and then a plateau from 100-cN to 200-cN force levels; meanwhile, in the adolescent group, an initial increase in cytokine activities occurred when forces increased from 50 to 150 cN, and then a plateau from 150- to 200-cN force levels.

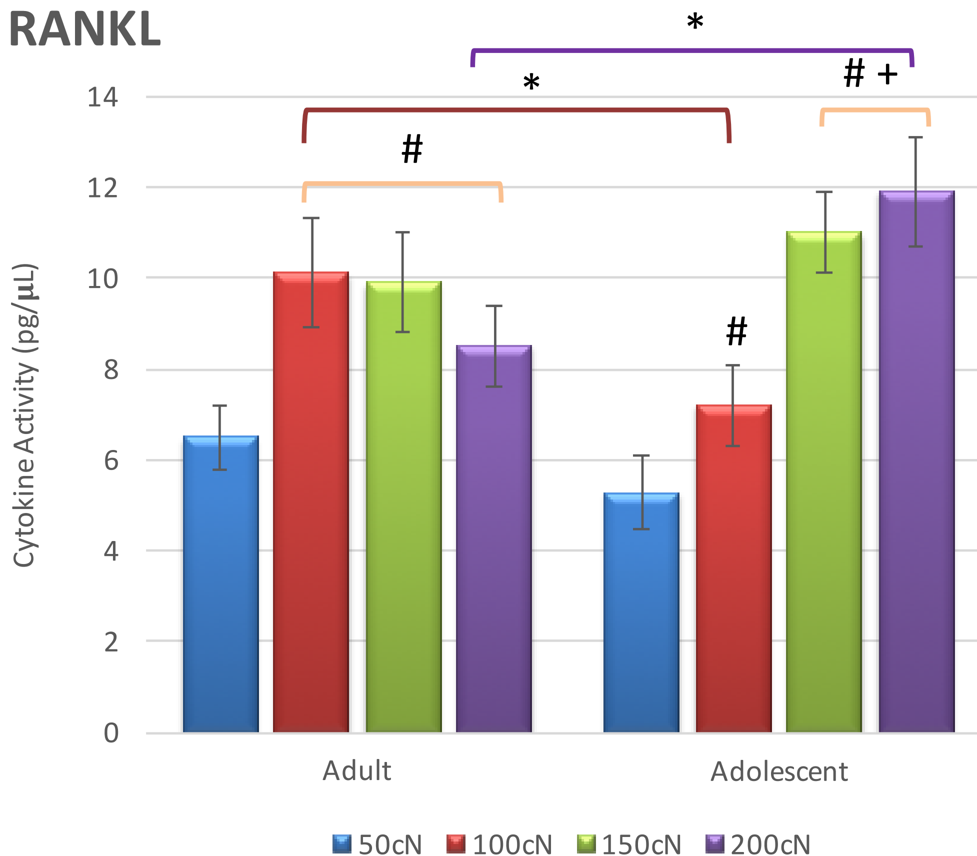


Figure 6-6. Higher level of osteoclast marker RANKL was expressed in adults in response to same magnitude of orthodontic force. GCF was collected from the distolabial gingival crevice of maxillary canines one day after retraction and mean concentration of RANKL was evaluated by a protein array. Each experiment was repeated 3 times, and the data was expressed as the mean ± standard deviation concentration in picograms per microliter (pg/µL) (#, significantly different from 50-cN group within the same age group; +, significantly different from 100-cN group within the same age group; *, significantly different between adolescents and adults who received the same force magnitude).

6.4.4. Rate of Tooth Movement

The rate of canine retraction in the first 28 days was measured on the study models at 3 landmarks: incisal, middle, and cervical thirds of the crowns (Fig 6-4, B).

In the adolescent group, when compared with 50-cN group, the amount of canine retraction increased significantly in 150- and 200-cN groups by 1.56- and 1.60-fold, respectively ($p < 0.05$; Fig. 6-7). However, the difference in the level of concentration was not significant between subjects who received 100- and 150-cN groups, or between 100- and 200-cN groups ($p > 0.05$).

In the adult group, there was no significant difference in rate of canine retraction observed among all force groups ($p > 0.05$), although there was a clinically noticeable increase from 50 to 100 cN.

When compared the amount of movement in first 28 days between adolescents and adults who received the same magnitude of force, adolescents appeared to have higher rate of movement in all force groups; however, the difference was not statistical significant ($p > 0.05$).

The result has demonstrated that adolescents reach saturation of at a higher magnitude of force than adults, as the rate of tooth movement in adults did not increase with higher magnitude of force, but adolescents did.

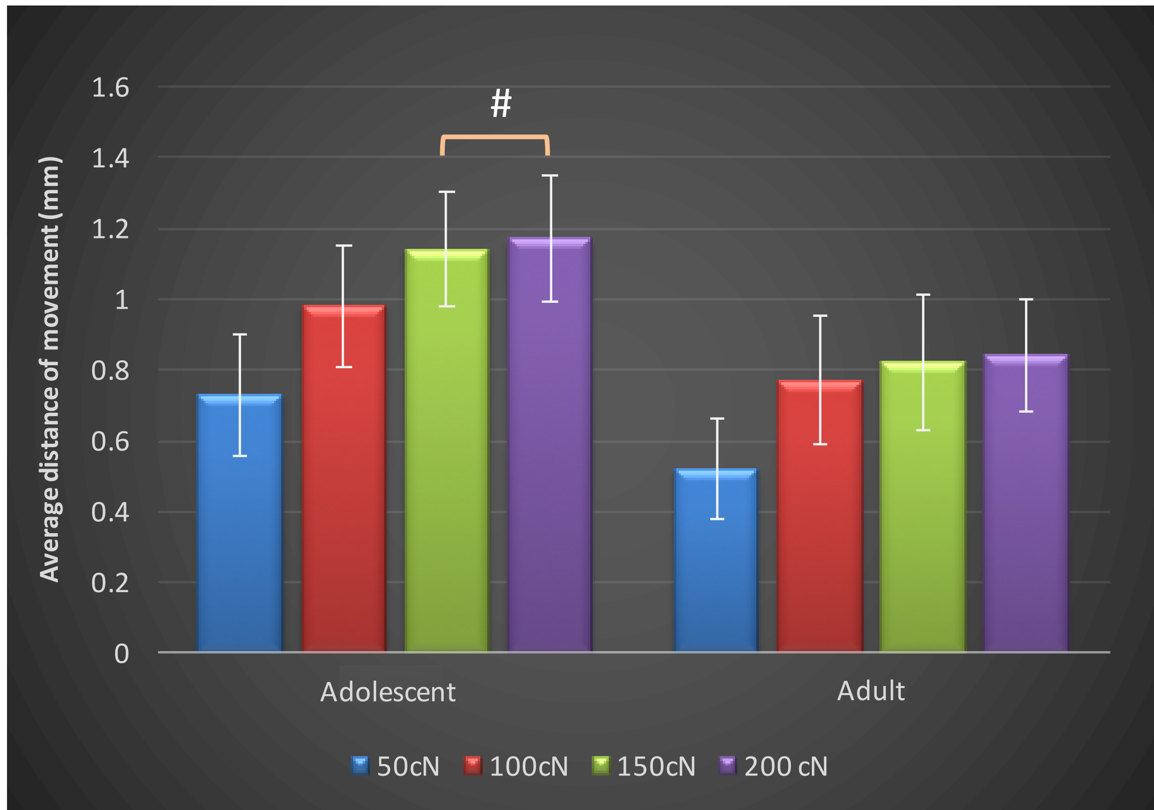


Figure 6-7. Comparison of amount of canine retraction using different magnitude of force between adolescents and adults in 28 days. The graph shows the means and standard deviations of the amount of tooth movement in millimeters measured at 3 landmarks (Fig. 6-4) for both adolescent and adult groups after 28 days of canine retraction. In the adolescent group, when compared with 50-cN group, the amount of canine retraction increased significantly in 150- and 200-cN groups. Each value represents the mean ± standard deviation movement of all subjects in their respective age group (#, significantly different from 50-cN group within the same age group, $p < 0.05$).

6.5. Discussion

To obtain further insights into the saturation of biological response among different individuals, we examined the activity of inflammatory markers and its correlated rate of tooth movement in response to different force magnitudes in two age groups. Our previous studies have shown saturation of biological response to higher magnitude of force, and the difference in biological response to an identical force magnitude between two age groups. In this present study, we have demonstrated the existence of biological saturation in humans, and the saturation point varies among different individuals.

Many factors could affect one's biological response to orthodontic force, however, in this particular study, we chose "age" as the single variable to represent the difference among individuals, as this is a factor that is easier to dichotomize with less ambiguity. Our results demonstrated saturation of biological response to higher magnitude of orthodontic force in both age groups. At lower level of force, there is an initial increase in the activity of inflammatory cytokines in both age groups, and then it reaches saturation with increased force magnitude. The saturation point is different between two age groups: while in adults, the saturation occurs between 100- to 150-cN force levels, in adolescents such saturation occurs between 150- to 200-cN force levels. Such difference implies that younger individuals have higher saturation points than mature individuals. We believe it is the first study in orthodontics describing and comparing such phenomenon and difference.

The observation of different saturation points among individuals also resonates and further explains the rationale for choosing appropriate force magnitude in our previous clinical study, as we descried in Chapter 5. We selected force magnitude of 50 cN for canine retraction in that study, to avoid the effect of saturation we revealed in both our animal study and the present

clinical study. In the present study we observed that adults reach saturation point at lower force magnitude than adolescents; in other words, while the biological response to 50-cN force is higher in adults than in adolescents who received the identical force, the biological response to 150- or 200-cN force is higher in adolescents than in adults. As a result, if in last clinical study we had chosen a much higher magnitude of force that had surpassed the saturation point of either or both age groups, we would have observed false positive or false negative results and drawn a misleading conclusion. Such issues have been noticed during my literature review on this subject, as some of the studies applied much higher magnitudes of force to evaluate the biological responses among individuals (Kawasaki et al. 2006).

The observation period of rate of movement in this study was limited to 28 days after retraction, as during the process of bone remodeling, bone mineral density and microenvironments surrounding the tooth constantly change over the course of tooth movement in a longer term. Therefore, as bone remodeling progresses, biological response within the same individual is unlikely to remain the same over time. Likewise, saturation point could change over time within the same individual. The difference in biological responses and rate of tooth movement in response in changing microenvironment over a course of time require further research, and is currently being investigated in our laboratory. On the other hand, the downside of such short-term observation is that the difference between age groups in rate of tooth movement was not obvious enough to reach statistical significance. However, the result still demonstrated a trend of faster movement in adolescents than adults in general; in addition, both age groups demonstrated saturation of rate of tooth movement, with a lower saturation point in adults and higher in adolescents — which corresponded well with our findings at the molecular level.

Although tooth movement is the desired result of the biological response to orthodontic forces, it may not necessarily be a precise representative of the biological response that cause tooth movement, since many other factors can affect the amount of tooth movement (Dudic et al. 2013; Krishnan and Davidovitch 2009; Ren et al. 2003a). Though some studies have shown that application of higher forces does not increase the rate of tooth movement (Quinn and Yoshikawa 1985; Ren et al. 2004), others have argued the opposite (Yee et al. 2009). This paradox is explained by the inappropriate use of tooth movement as a measure of the effect of force magnitude on the rate of tooth movement.

While majority of the studies focused on evaluating the difference in the rate of tooth movement to different magnitudes of force in animals (Gonzales et al. 2008; Pilon et al. 1996; Ren et al. 2003a; Storey and Smith 1952; Van Leeuwen et al. 2010; van Leeuwen et al. 1999; Yee et al. 2009) and humans (Boester and Johnston 1974; Iwasaki et al. 2000; Luppanapornlarp et al. 2010; Quinn and Yoshikawa 1985; Ren et al. 2003a), little evidence is available regarding the differences in terms of their biological responses to different magnitudes of orthodontic force, especially in humans (Iwasaki et al. 2001; Luppanapornlarp et al. 2010). Previous studies comparing biological responses in GCF to different magnitudes of force lack consistency in appliance design, protocols, type of tooth movement, observation period, and biomarkers evaluated. Therefore, this field warrants further research using a reliable and reproducible method including using a constant and continuous force, and carefully selected force magnitude, age range and observation period. The result of our present study has shed light not only on the difference in biological responses between juveniles and adults to orthodontic force, but a different pattern of response to higher force magnitude.

The presence/expression of regulatory proteins in the GCF has been recognized as a promising non-invasive diagnostic tool for monitoring orthodontic treatment outcome (Ren and Vissink 2008) since it has been shown that GCF may reflect the immune and inflammatory reactions arising from the application of orthodontic force (Kapoor et al. 2014; Ren et al. 2007; Ren et al. 2002; Uematsu et al. 1996). The inflammatory markers we selected for analysis in this study were based on their known functions, and the results from our previous studies (Alikhani et al. 2013; Teixeira et al. 2010).

Our present study demonstrates that the activities of cytokines (IL-1β and RANKL) and chemokine (CCL-2) significantly elevated during orthodontic tooth movement, and responded immediately after orthodontic loading. Since all these molecules play significant roles in recruitment and activation of osteoclast precursor cells, one may assume that increased activities of these factors should be accompanied by higher osteoclast activation and therefore a higher rate of tooth movement (Fuller et al. 2006; Jimi et al. 1996; O'Brien et al. 1999; Suzawa et al. 2000). However, it is partially true as the difference within and between individuals should be evaluated as two different entities. While higher level of these inflammatory marker results in greater rate of OTM within the same individual, higher level of inflammatory markers does not guarantee greater rate of OTM when it comes to a comparison between different individuals, as we demonstrated in last two Chapters in this dissertation. Similarly, saturation point cannot be predicted based on that of another individual. On the other hand, in the same individual, if there is no significant difference in biological response to two different magnitude of force, then we do not expect significant difference in the rate of OTM either.

Thus, while increasing the force magnitude does not overcome this limitation, other methodologies, such as MOP, is sometimes necessary to enhance the biological response, in turn

improving treatment efficiency and decreasing adverse tissue response in certain clinical scenarios. It is advisable that since saturation point is found to be lower in adults, acceleration techniques are more justified to be applied on adults than juveniles.

Many factors other than age could affect the rate of tooth movement and warrant further research. Poor oral hygiene, periodontal disease, advanced alveolar bone loss, systemic diseases, and anti-inflammatory medications can affect the rate of tooth movement significantly (Bartzela et al. 2009; Knop et al. 2012; Okamoto et al. 2009). To minimize the influence from these factors, we set clear exclusion criteria, as summarized in Table 6-1, and the subjects were able to maintain good oral hygiene.

Lastly, the concept of "an optimal orthodontic force" has been the subject of investigation for years. The concept has been evolved over the last 80 years. The first definition was proposed in 1932 (Schwarz 1932), "the force leading to a change in tissue pressure that approximated the capillary vessels' blood pressure, thus preventing their occlusion in the compressed periodontal ligament." According to this definition, forces well below the optimal level cause no reaction in the PDL. Forces exceeding the optimal level would lead to areas of tissue necrosis. Other definitions have been proposed, "the lightest force capable of bringing about tooth movement (Oppenheim 1942)," and observed cell-free zones within the pressure side even in cases where light forces were applied (Reitan 1967). The current concept of optimal force is based on the hypothesis that a force of a certain magnitude and temporal characteristics (continuous *vs.* intermittent, constant *vs.* declining, etc.) would be capable of producing a maximum rate of tooth movement without tissue damage and with maximum patient comfort. The optimal force for tooth movement may differ for each tooth and for each individual patient (Krishnan and Davidovitch 2006; Proffit 2013; Ren et al. 2003a). Earlier studies (before 1980) on this subject

focused on efficiency of OTM by using light vs. heavy force, and later studies focused on histological and cell biological changes, or side effects such as root resorption. The appropriate forces for tooth movement of human teeth reportedly range from a force as light as 18 g to one as heavy as 1515 g (Hixon et al. 1970; Iwasaki et al. 2000; Ren et al. 2003a). This argument still exists, and no evidence-based optimal force level can be recommended in clinical orthodontics (Luppanapornlarp et al. 2010).

Although defining optimal force is not the main concern of our study, it's worth mentioning that the concept of optimal force can be re-invented from a biological response point of view. From this perspective, an optimal force is defined based on the saturation point of that particular patient and situation — a force that induces biological response close to reaching its saturation, and an increase of the force cannot further induce more inflammatory response. According to our studies, since biological response dictates the rate of bone remodeling thus rate of OTM, any force increase beyond this point does not increase rate of OTM either (Fig. 6-8), therefore the rate of OTM is maximized at this force magnitude. The difference in saturation point between adults and adolescents implied that lower magnitudes of orthodontic force should be utilized in treating adult patients to optimize the amount of tooth movement and avoid adverse tissue responses.

In summary, the result of our animal study suggests using the level of the inflammatory response as a predicative factor for the rate of tooth movement and optimal force. There is difference in saturation of biological response and thus optimal force for OTM among individuals. Therefore, predicting an individuals' saturation point based on another individual would be erroneous without considering individual variables such as age; one should always compare the level of inflammatory markers and saturation point within the same individual.

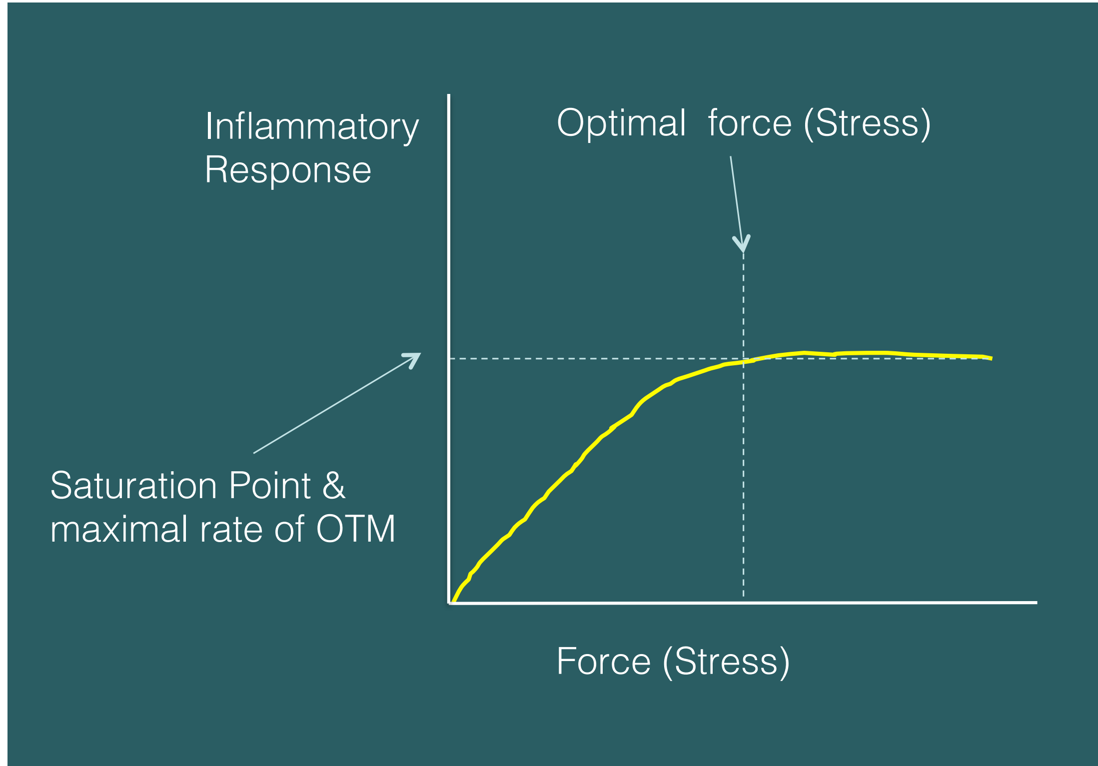


Figure 6-8. From biological response point of view, optimal force can be defined according to saturation point. From this perspective, an optimal force is defined based on the saturation point of that particular patient and situation — a force that induces biological response close to reaching its saturation, and an increase of the force cannot further induce more inflammatory response. Since biological response dictates the rate of bone remodeling thus rate of OTM, any force increase beyond this point does not increase rate of tooth movement either.

6.6. Conclusions

1. Saturation of biological response to higher magnitude of orthodontic force exists in humans.
2. Saturation point varies among different individuals. Adolescents reach saturation point at higher magnitude of force than adults; therefore, while the biological response is higher in adults at lower force magnitudes, it can exhibit the opposite results when applying higher force magnitudes.
3. The results of this study implied that the optimal force should be defined based on each individual's biological response. Lower magnitudes of orthodontic force should be utilized in treating adult patients to optimize the amount of tooth movement and avoid adverse tissue responses.

Chapter 7. Summary and Future Research Directions

Our null hypothesis “there is no difference in the level of cytokine activation within individuals or between individuals receiving different magnitudes of orthodontic forces” has been rejected based on the results of our animal and clinical studies. First, in our animal study we demonstrated that after a certain magnitude of force, there is a saturation in the biological response, where higher magnitude of force does not increase inflammatory markers, osteoclasts, nor amount of tooth movement. Therefore, higher forces to accelerate the rate of tooth movement are not justified. Second, we demonstrated that the level of biological response varies among individuals to the same magnitude of force. Therefore, one should compare the level of biological response within the same individual. Third, saturation of biological response to higher magnitude of orthodontic force exists in humans, and saturation point varies among individuals. Adolescents exhibit higher saturation point than adults; therefore, the optimal force should be defined based on each individual’s biological response. Lower magnitudes of orthodontic force should be applied in treating adult patients to optimize the amount of tooth movement and avoid adverse tissue responses.

This research has a strong of clinical implication. Although increasing the magnitude of orthodontic force increases inflammatory marker levels, osteoclast recruitment and formation, alveolar bone resorption, and the rate of tooth movement, there is a force level above which we cannot stimulate these biological responses any further. The magnitude of cytokine release that can be induced by orthodontic forces has an upper limit and consequently the osteoclast activity initiated by orthodontic forces has a saturation point. Keep increasing force magnitude will not provide more clinical advantage but only increasing the risk of adverse effect. Therefore, while

increasing the force magnitude does not overcome this limitation, other methodologies, such as MOP, is sometimes necessary to enhance the biological response, in turn improving treatment efficiency and decreasing adverse tissue response in certain clinical scenarios.

To our knowledge, this is the first study that identified and described "saturation of biological response" to orthodontic mechanical stimuli, and demonstrated different biological response among individuals from a biological standpoint instead of physical phenomenon. This project has improved our fundamental understanding on biological response to orthodontic force, and lends itself to further exploration on the biological response in a longer period of time. Does saturation point change after tooth movement has occurred? If it does change, shall it increase or decrease? Does optimal force also change during the course of movement? Do such changes occur in both adolescents and adults? If so, does the amount of change similar or one group change more than the other? What is saturation point for different type of tooth movement? Is saturation point different between en masse *versus* individual tooth movement? What are optimal forces for different teeth and different types of tooth movement? It should be emphasized that although saturation point was discussed based on different magnitudes of force due to limitations of both animal and human models, ideally, it should be defined based on stress, taking into account the force distribution on root surface. This may unify the saturation point for different types of tooth movement.

Ultimately, by exploring this intriguing topic more thoroughly and comprehensively, it will help clinicians to make optimized and customized treatment decisions for their patients, and improve the treatment efficiency by reinventing the current treatment system. It can further inspire revolutionary treatment system and modalities in orthodontics and help evolve our specialty.

Appendix

Abbreviation Glossary

CCL	Chemokine ligand
cN	centinewton
ELISA	enzyme-linked immunosorbent assay
GCF	gingival crevicular fluid
IGF	insulin-like growth factor
IL	interleukin
μCT	X-ray microtomography/micro-computed tomography
MCP	monocyte chemotactic/chemoattractant protein
M-CSF	macrophage colony-stimulating factor
MOP	micro-osteoperforation
NSAID	non-steroidal anti-inflammatory drug
OH	oral hygiene
OPG	osteoprotegerin
OTM	orthodontic tooth movement
PCR	polymerase chain reaction
PDL	periodontal ligament
PG	prostaglandin
RANK	receptor activator of nuclear factor κ B
RANKL	receptor activator of nuclear factor kappa-B ligand, a.k.a. TRANCE
RANTES	regulated on activation, normal T cell expressed and secreted
SEM	standard error of the mean
TNF	tumor necrosis factor
TRAP	tartrate-resistant acid phosphatase

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