The effect of two wattages of low-level laser therapy on orthodontic tooth movement

by

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Abstract

The use of low-level laser therapy (LLLT) to accelerate orthodontic tooth movement (OTM) has generated mixed outcomes due to the variability of parameters used. The aims of this study were to test for differences in OTM, gene expression using RT-qPCR, and histological changes of molars exposed to two different wattages at the same energy density.

A 10g force was applied to molars of three rat groups: CT (no laser), EX-500 (500 mW)and EX-1000 (1000 mW).

There were statistically significant differences in OTM between EX-500 and CT groups and *RANKL* and *MMP-13* expression levels between CT and laser groups. Histologically, 56% of the EX-1000 revealed early dysplastic changes.

Both wattages of laser therapy increased remodeling in the PDL that may or may not translate into increased OTM. Future studies should focus on elucidating possible biphasic responses of LLLT before it can be offered as a consistent modality for accelerating OTM.

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Chapter 1
Introduction

The current study was conducted to better understand one specific parameter for the use of low-level laser therapy (LLLT) as a modality to accelerate orthodontic tooth movement (OTM). A background of laser, its mechanisms of interactions, and its uses in clinical dentistry and orthodontics will be provided below. In addition, some basic biological concepts of OTM will be explained in detail, following which a review will be conducted on published literature on the use of LLLT and its use in accelerating OTM in both human and rat studies. An introduction will also be given for the Ardnt-Schultz Law, around which the aim of this study was based.

1.1 History of Lasers

Today, lasers (**L**ight **A**mplification by **S**timulated **E**mission of **R**adiation) are commonplace in our everyday lives, yet it was not that long ago when laser energy was just an idea. Light therapy dates back to Egyptian times when it was known as solar or ultraviolet (UV) therapy.[1] A more complex understanding of light came with Einstein's postulates of quantum theory in 1917.[2] The first principle is that light travels in discrete packets of energy known as photons. The second principle is that atoms exist in a ground state and by adding thermal, electrical, or optical energy to those atoms they can become excited to higher energy levels. Upon excitation, these atoms release energy spontaneously in the form of photons or electromagnetic waves as they return to their ground state.[2] The concept of laser evolved from the discovery that this excitation could be amplified through the use of mirrors and focused into a beam to do useful work.[2] From this, physicists began to experiment with the possibility of amplifying stimulated emission and in 1957 Gould coined the acronym "laser".

The first laser, a solid ruby laser, was first made by Maiman in 1960.[2] After that, IBM Laboratories came out with the first uranium laser followed by the helium-neon (He-Ne) laser by Bell Laboratories. It was then followed by the neodymium-doped yttrium aluminum garnet (Nd:YAG) semiconductor laser and CO_2 laser, developed in 1964, the same year as the argon laser.[3,4] The first surgical laser designed for dentistry was in 1989 and it was not until 1997 that the United States received FDA approval for Er:YAG laser use on dental hard tissues.[5]

1.2 Description of Lasers

Laser light is a type of electromagnetic energy that exhibits the duality of both a particle and wave. The laser consists of three parts: an excitation source (a flash lamp strobe device, an electrical current, or an electrical coil)[6], an active medium, and an optical resonator.[3,5] Lasers are classified according to the media used, such as gas (nitrogen-CO_2, He-Ne, CO_2), liquid dyes (rhodamine 6G), semiconductor materials [gallium arsenide (GaAs), indium gallium arsenide (InGaAs), or gallium nitride (GaN)], glasses (silicate or phosphate glasses doped with laser-active ions or artificial gemstones/crystals as a [yttrium-aluminum-garnet (YAG)], and moving charged particles (free-electron laser).[3] The optical resonator is composed of two mirrors on either side of the medium cavity that the laser light bounces off of to amplify the power and escapes through the partially reflective mirror at a given power output.[3]

Meister described lasers as transforming "low quality" energy into "high quality" energy such that light, kinetic, or electrical energy becomes transformed into optical energy.[3] Once emitted, the photons have three characteristic properties: 1) they move at the speed of light, 2) they have a certain amplitude (expressed as joules), and 3) they have a specific wavelength.[3] Amplitude describes the amount of energy the wave carries, meaning the higher the amplitude, the higher the energy. The wavelength is described as the horizontal difference between corresponding points on a wave so that the longer the distance between them the longer the wavelength.[5] What distinguishes laser light is that it is only generated in one colour (monochromatic) and the waves are all identical in shape, size, and direction (coherent) making this energy source uniquely efficient and capable of doing useful work.[4,5,7] Each type of laser produces a specific wavelength dependent on its active medium that allows for different interactions with different tissue types. Laser light is delivered to the desired tissue through either a fiber-optic cable, a hollow waveguide, or an articulated arm.[5]

1.3 Laser-Tissue Interactions

When laser energy is directed towards a tissue there are three outcomes that can interrupt its transmission through the target tissue:[8]

Reflection: The return of electromagnetic radiation off the surface upon which it is incident or a redirection of the beam with no effect on the target tissue.

Absorption: The ratio between absorbed and incident intensities, a value based on the medium.
Scattering: The dispersion of the incident light upon entering a given medium resulting in a weakening of the delivered energy and potential transfer of heat to adjacent tissues. The amount of loss is dependent on the type of material and incident wavelength.[8]
It should be noted that while the most desired interaction is *absorption* we cannot prevent all of these interactions from occurring simultaneously.[5]

The first law of photobiology states that for light to have an effect on a biological system the photons of a particular wavelength must be absorbed by electronic absorption bands of a chromophore or photoacceptor within the system of interest.[1] The principal chromophores of soft tissue are hemoglobin and melanin, which have high absorption bands below the wavelengths of 600nm. There is discussion in the literature in regards to an "optical window" where the ideal soft tissue laser would have the highest absorption for hemoglobin and melanin with low absorption of hydroxyapatite in order to be used safely within close proximity of teeth.[1] This optical window covers the red and near-infrared wavelengths (600-950nm) for greatest tissue absorption.[1]

The number of tissue-laser interactions are numerous and dependent on tissue characteristics and laser parameters. These laser parameters include wavelength [nanometers (nm)], exposure time [seconds (s)], power [milliwatt (mW)], energy [joules (J=Wxs)], focal spot area (cm^2), power density (W/cm^2), and energy density (J/cm^2).[6,8] The most important factors for phototherapy are wavelength (to target the biological system of interest), wattage (which determines the amount of energy delivered) and laser exposure time (that will influence both energy and energy density).[3,8] Altering these parameters can result in different interactions that occur within energy densities of 1-1000J/cm^2 in a linear relationship and range from photochemical to photodisruptive interactions.[2] Therefore, lower power density applied for a relatively longer duration will result in photochemical interactions occurring, where light induces a chemical effect within macromolecules or tissues. These interactions occur most commonly with a red dye laser or a diode laser and are used for healing and repair processes.[8] Photochemical interactions can be described as photodynamic therapy (PDT) and photobiostimulation. At higher power density, thermal interactions occur, resulting in an increase in the local temperature of the tissue in processes such as coagulation, vaporization, carbonization, and melting.[8] At still higher power

densities, plasma ionization occurs in exposed tissues by a process known as photoablation.[8] Plasma-induced ablation (PIA) happens when power densities exceed 10^{11}W/cm^2 and is the most commonly used laser application in dentistry.[9] During photoablation, the tissue decomposes in a clean and predictable manner through vaporization and super heating of tissue fluids without thermal or mechanical damage. Photoablation falls under the photodecomposition group and can occur in both hard and soft tissue and is used in caries therapy.[8]Photodisruption/photomechanical interactions allow the creation of minimally invasive surgery that results in tissue splitting from mechanical forces.[8] Tissue removal occurs through laser light and photo acoustic interactions. This interaction is different from PIA since ablation is due to mechanical forces versus ionization, respectively.[8]

1.4 Biological Mechanisms for Low-Level Laser Therapy

Of interest to the current study is the biostimulatory effects of laser. Photobiostimulation is defined as the effects resulting from the use of lasers at very low powers (1-500mW) and fluencies (1-10 J/cm^2). Photobiostimulation is also described under numerous terminologies in the literature, e.g., low-level laser therapy (LLLT), low energy laser therapy (LELT), and low intensity laser therapy (LILT). Presently, LLLT is also known as cold laser, soft laser, biostimulation, photobiomodulation, photostimulation, photobiostimulation, and biophoto-modulation.[1]

LLLT can be defined as energy densities below those required for ablation, cutting, and thermally coagulating tissue.[3] This involves low levels of energy (mW) absorbed by a target tissue to produce changes in chemical and physical properties with little or no temperature change.[10] The most common light sources for photobiostimulation are the He-Ne laser, argon laser, and diode laser.[11] The first demonstration of laser photobiostimulation was by Meister in 1967 when he conducted an experiment to determine if laser radiation would cause cancer growth in mice.[1] The mice did not develop cancer but instead had hair growth occur more quickly than the control group.

Biostimulatory interactions have gained significant usage in both the medical and dental fields.[8] Three main contributions of LLLT in medicine are:[1]

1) Wound healing, tissue repair, and prevention of tissue death,

2) Reduction of inflammation in both acute injuries and chronic diseases from its associated pain and edema, and
3) Relief of neurogenic pain and neurological problems, problems present in dentistry as well, thereby explaining the use of lasers in dentistry.

Although LLLT has been used for over 40 years to promote healing, reduce pain and inflammation, and prevent tissue death, its mechanisms have still not been fully elucidated.[12] Several mechanisms have been proposed with no consensus on a single mechanism (Figure 1). Photobiostimulation has been proposed to stimulate mitochondrial cytochrome c oxidase, the primary photo-acceptor in the wavelength range of 600-950 nm, resulting in increased cell metabolism (via ATP, cAMP and Ca^{2+}).[1,13-15] ATP is responsible for the regulation of secondary messengers, like cAMP and Ca^{2+}. These mediators are involved in many processes in the body including muscle contraction, blood coagulation, signal transfer in nerves, and gene expression.[1]

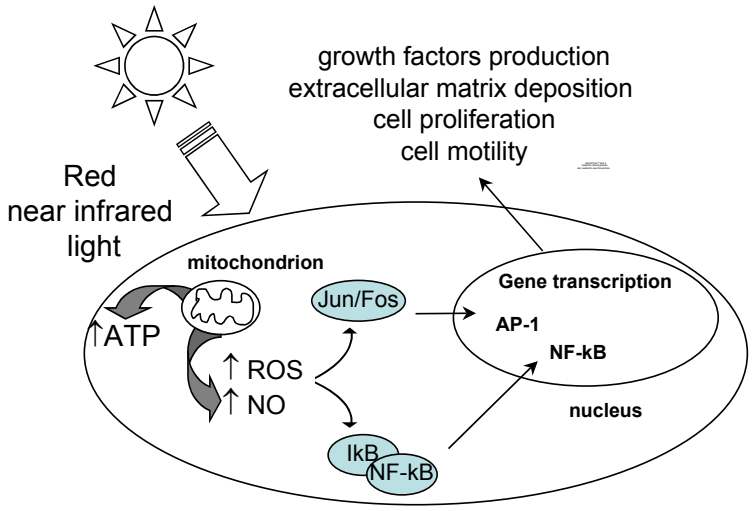

Figure 1 Cell signaling pathways induced by LLLT. Reproduced from Hamblin. Mechanisms of low level light therapy. Proc. of SPIE 2006;6140 with permission[1]

A "singlet-oxygen hypothesis" has also been proposed. In this hypothesis, molecules like porphyrins and some flavoproteins are activated upon laser application and interact with oxygen to create reactive singlet oxygen to eliminate energy deficits and increases overall cell metabolism.[1,16] Reactive oxygen species (ROS) are believed to be involved in the signaling

pathways following photon initiation resulting in activation of nuclear factor kappa B (NF-kB), a transcription factor and a purported sensor for oxidation stress. Chen et al.[12] identified that low levels of near-infrared laser light can activate NF-kB in mouse embryonic fibroblasts using a diode laser. Alongside an increase in ROS, there is also an increase in antioxidant production.[17]

A complementary hypothesis is the "redox properties alteration hypothesis". Photobiostimulation is believed to change the overall cell redox potential towards that of greater oxidation and an increase in the ability of cells with a lower resting intracellular state to activate transcription factors.[1,16] Lastly, it has been hypothesized that there is a direct impact on RNA and DNA synthesis and replication, protein synthesis and overall cell metabolism through increased Na^+/K^+ pump activity and intracellular Ca^{2+} production.[13,18] While these mechanisms have been published separately, it is possible they work simultaneously to affect several levels of cellular activity during photobiostimulation. It is important to note that only a small number of photons are needed to produce a small change in the cellular redox state to result in a large biological effect.[17]

1.5 Application of Laser in Dentistry

Historically, laser use began in the field of ophthalmology for retinal repair.[2,8] Presently, lasers are an indispensable tool for diagnosis and therapy in many areas of medicine. Dentistry was the second field to clinically adopt the use of laser, with both intra- and extraoral applications.[8] Depending on the particular dental need, lasers with specific parameters are selected. In terms of wavelengths, dental uses fall within the range of 488 to 10,600nm.[6] In dentistry, laser therapy has been used in caries detection in preventative dentistry, desensitizing sensitive teeth and curing composite resin in restorative dentistry, sterilization of infected root canals in endodontics, sterilization and reduction of pocket depths in periodontics, and extensively used for soft tissue procedures in oral surgery.[8-10,19] High-level laser therapy is usually used in hard tissue modification, specifically in the removal of caries. In addition, CO_2 lasers have been used for the management of malignancies, premalignant lesions, and leukoplakias because of their high absorbance for water resulting in rapid tissue ablation in a controlled and predictable manner with hemostasis.[8]

1.6 Application of Laser in Orthodontics

Both high and low intensity lasers are being used in orthodontics. Laser has several benefits for an orthodontic office. It allows the orthodontist to perform procedures that might otherwise be referred to other specialists and its use resulting in minimal pain has been shown to diminish dental fears for patients leading to improved cooperation.[20] Studies are being conducted with various lasers to assess their use in enamel conditioning before bonding and for improving enamel's resistance to acid to avoid potential demineralization and white spot lesions.[21,22] Laser is also being investigated as a potential replacement of hydrofluoric acid in bonding to porcelain, thereby avoiding gingival burns and the need to repolish porcelain at debonding.[23] High-level lasers are also being used in soft tissue applications such as gingival recontouring and reshaping, removal of excess tissue from gingival hyperplasia, gaining access to labial tooth surfaces for bracket placement, removal of redundant tissues created by space closure or from poor oral hygiene, removal of opercula on second molars, fiberotomy, and frenectomy.[3,9] The advantages of laser compared with scalpel in these situations is minimal bleeding, reduced postoperative pain and no swelling.[24] The LLLT applications investigated thus far include pain reduction after orthodontic appliance placement, pain management of temporomandibular joint disorders, bone regeneration after rapid palatal expansion, and, of greatest interest, increased orthodontic tooth movement (OTM).[3,25,26]

1.7 Orthodontic Tooth Movement

The biology behind orthodontic tooth movement (OTM) is a complex interplay of cellular and molecular changes that begins when a force is applied to a tooth. There are two theories of bone remodeling that are widely accepted: Pressure-Tension theory and Piezoelectric theory.[3,27] When a force is applied to the tooth it is displaced within the periodontal ligament (PDL) resulting in compression in some areas and stretching in others. These areas experience changes in blood flow that stimulate the release of biologically active mediators resulting in an increase of cellular activity and differentiation. It is also thought that bone bending creates an ionic change in the crystalline structure that translates into electrical currents.[27] These changes in the periradicular tissues stimulate multinuclear giant cells, osteocytes, osteoblasts, osteoclasts, and fibroblasts that allow for OTM to occur.

In humans, OTM occurs in three phases. There is an initial phase that occurs within seconds of force application as the tooth moves within the socket from the bending of the alveolar bone due to the incompressibility of the PDL fluids. This is followed by the lag phase that occurs within hours-2 days after force application. During the lag phase, there are changes to tissue oxygen levels resulting in the release of chemical mediators. Depending on the degree of blood vessel occlusion during this stage, there may be low rates of tooth movement or no displacement from the varying degree of hyalinization of the PDL. During the postlag or acceleration phase (2-14 days after force application) there is a continuous or sudden increase in tooth movement from cells recruited in the adjacent bone marrow depending on if hyalinization of tissue on the pressure side has occurred.[28] Thus, little tooth movement occurs in the early stages of OTM as it is dominated by extracellular matrix remodeling which involves inflammatory mediators (interleukins, tissue necrosis factor alpha, and prostaglandins), matrix-metalloproteinases (MMPs), and integrins. Cells responsible for bone remodeling are being recruited in the early stages. In the later stages bone resorption and deposition occur through the RANK/RANKL/Osteoprotegerin and RUNX2 signaling pathways.[29] Additional animal studies have shown that under lighter forces the amount of tooth movement observed may follow a more linear rate within the first day that is similar to physiologic movement.[30,31] With fibroblasts, osteoblasts, osteoclasts, numerous inflammatory modulators, and transcription factors involved in OTM there are a number of potential targets for increasing its rate.[32,33]

Several chemical agents and physical stimuli have previously been reported to increase OTM. Prostaglandin E2 (PGE_2), 1,25-dihydroxyvitamin D, parathyroid hormone, electromagnetic fields, vibration, electrical currents, and LLLT have been identified as mediators in accelerating tooth movement (Figure 2).[34,35] The difficulty with chemical agents is that they can have a negative systemic effect on the patient and their routes of administration are algesic.[26] Discovering the ability to accelerate bone remodeling while avoiding harmful systemic or local effects on the periodontal tissues resulting in faster tooth movement is of significant interest to patients and orthodontists.[35]

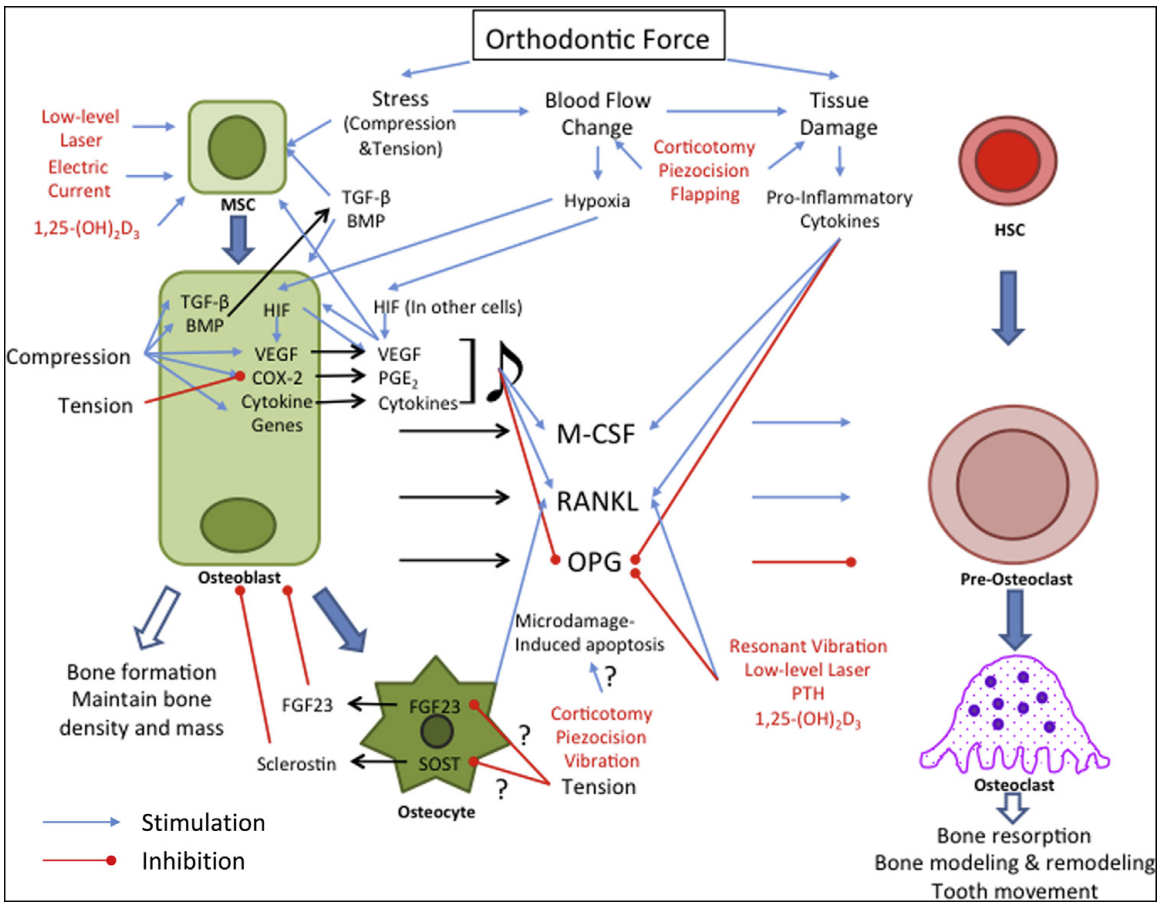

Figure 2 Summary of cellular and molecular mechanisms underlying accelerated orthodontic tooth movement. Methods to accelerate orthodontic tooth movement are shown in red. Blue arrow, Stimulation; red blunted arrow, inhibition; MSC, mesenchymal stem cell; HSC, hematopoietic stem cell; HIF, hypoxia inducible factor; FGF, fibroblast growth factor. Reproduced from Huang H. et al. (2014) with permission.

1.8 Biological Effects of LLLT on OTM

The cellular responses assessed *in vitro* with LLLT are broadly classified under increase in metabolism, migration, proliferation, and increases in synthesis and secretion of various proteins.[1] To characterize if LLLT could be used for photobiostimulation of OTM several *in vitro* experiments have been conducted. Yu et al[36] found a significant increase in basic fibroblast growth factor after keratinocytes and fibroblasts were irradiated with 0.5-1.5J/cm^2 HeNe laser. This was confirmed by others reporting an increased proliferation of fibroblasts and increased collagen type I production.[37,38] *In vitro* studies have also shown upregulation of RANK/RANKL and c-Fms gene expressions, which are known mediators for osteoclast activity,[39-41] demonstrating an enhanced proliferation of osteoblast-like cells.[42] Investigations by Yamaguchi et al.[43] found that there was an acceleration of bone remodeling found in the rat after LLLT

through stimulation of MMP-9, cathepsin K, and integrin alpha(v)beta(3) expression during OTM. Abi-Ramia et al.[44] reported that the biomodulating effects of LLLT include fibroblast proliferation, collagen synthesis, and organization of collagen fibers. They also found that LLLT in combination with OTM resulted in increased vascularization, which may allow for accelerated pulp tissue repair in rats.[44] Yoshida et al.[45] investigated the effect of LLLT on tooth movement and found that at each time point in their investigation their irradiated group had significantly greater tooth movement compared to their control group. This may be attributed to improved turnover of connective tissue by way of increased expression of fibronectin and collagen type I[25,34,46] as well as stimulating osteoblast and osteoclast cell proliferation.[47] LLLT significantly increased osteoclastic but not osteoblastic activity during the initial phases of tooth movement and that the osteoclastic activity was dose-dependent.[48] Most of the animal studies concluded that LLLT increased tooth movement by stimulating bone remodeling, yet there were a few that found no significant difference between irradiation and controls.[26] These and other studies of similar nature have supported the potential for LLLT to influence OTM in humans. Several animal studies have been performed with diode lasers to accelerate the rate of OTM.[39,40,43-45,49,50] The rat model is the most commonly utilized animal but studies have also included monkeys, dogs, and cats.[26,51] Comparison among these studies has been rendered difficult due to the use of numerous different parameters between the studies: (a) *sample size*–ranging from 12-30 animals; (b) *energy output*- wattage ranges from 40-180mW; (c) *wavelength* - varies within the infrared region from 780 to 850 nm; (d) *orthodontic force levels* - in rats, ranges from 10-40g; in dogs from 85 and 150g, and, (e) *method of LLLT application* - varies in the number of application points and use of either pulsed or continuous wave operating modes.[52]

Overall, the majority of the animal studies showed a positive effect of LLLT application. Fold change appeared to be the most commonly used method for quantitating and comparing the amount of tooth movement. For example, 1.34 [45] and 1.5 fold [39] increases were observed in LLLT-exposed groups over a period of 21 and 7 days, respectively. However, while these findings were statistically significant, actual numbers were often absent and the *actual* difference in tooth movement between experimental and control groups were < 0.2 mm in many cases.[39,43,45,49] Interestingly, some studies have shown that the benefit to OTM in irradiated groups versus controls decreased over time.[39,43] For example, Yamaguchi et al. showed

differences of 2.0-fold increase at day 3, but by days 4 and 7, the increases had slipped to 1.9- and 1.3-folds, respectively.[43]

Three other separate studies, using different methodologies (e.g., fewer laser applications, extraoral application of laser, and higher orthodontic forces) found no significant difference between photobiostimulation and controls in affecting the rate of OTM.[35,47,53] Similar benefits of increased OTM were not seen in beagle dogs at 2 months of LLLT despite histological evidence suggestive of increased osteoclastic activity.[52,54]

Several clinical studies have been published analyzing the effects of LLLT on canine retraction in a split mouth design. Cruz et al.[55], Youssef et al.[56], and da Silva Sousa et al.[57] found significantly greater velocity of tooth movement with LLLT. Limpanichkul et al.[58] did not find an increase in tooth velocity. And while their total energy density was 25J/cm^2 compared with the previous three studies at 5J/cm^2 their area of application was smaller which may have accounted for their findings. Cruz et al.[55] found that their laser group demonstrated a 34% greater retraction rate relative to a control group, and Doshi-Mehta & Bhad-Patil[59] found that their irradiated group had an increased retraction rate of 1.3 times greater compared to their control group. The study by Doshi-Mehta & Bhad-Patil has been critiqued for inappropriate use of statistical analysis making their data difficult to accept with certainty. The lack of effect found by Limpanichkul et al.[58] and Kocoglu-Itan et al.[60] was hypothesized to be a result of an incorrect dose leading to reduced levels of arachidonic acid and PGE_2, a key mediator in osteoclastic activity. Dominguez et al.[28] conducted a clinical study assessing gingival crevicular fluid with LLLT to assess the levels of RANKL and OPG and found a slight improvement in OTM. They noticed a trend of increased tooth movement at the beginning of their observation period with a decrease to slower than their control group at 30-45 days. Overall, the laser group exhibited greater accumulated tooth movement with a change in the rate of movement that is similar to that observed in the animal studies by Yamaguchi et al.[28,43] Overall, these studies illustrate that although the dose dependent effect of LLLT has not yet been determined, LLLT has been shown to affect bone metabolism and OTM at the cellular, animal, and human levels without any harmful effects. Long et al. conducted a systematic review of interventions accelerating OTM and concluded from the three studies they included that it is safe but unable to accelerate tooth movement.[61] The reason for the inclusion of only three articles was due to the authors'

assessment of quality and bias. Contrarily, Jawad et al.[62] conducted a literature review and found LLLT to be beneficial for OTM.

Several systematic reviews have been published in regards to the animal studies published with varying outcomes. Torri & Weber published a literature review on the various protocols for LLLT and their effects on OTM.[26] They reported on six rat studies with the conclusions that three studies found LLLT application did increase the rate of OTM and that the other three may have been insignificant due to small sample size. Carvalho-Lobato et al. conducted a systematic review of OTM with LLLT with 10 rat studies.[51] Their conclusions are that it is possible to achieve the desired biological effect of accelerating OTM; however, there were studies not demonstrating any benefit.[51] This systematic review included an *in vitro* experiment and an animal experiment that did not include OTM data that some authors feel makes the conclusions less reliable.[63] The various conclusions are determined by which studies the authors choose to include and even at the systematic review level of evidence it is still uncertain as to whether LLLT is an effective method for accelerating OTM and what, if any, were the parameters that may be responsible for the negative outcomes reported. An important difference to note between human and animal studies is the amount of power and overall energy exposure of the subjects. A marked difference between the rat and human studies is the laser application power. Increased tooth movement was seen in humans at 20mW[55,64] and but not at 100mW[58] and, conversely, was seen in animal studies at 100mW[39,45,49] but not at 40mW.[53] Some of the discrepancies arise due to the large number or parameters that did not remain constant in all the experiments, as is mentioned above. The authors suggest that further studies are warranted to standardize these parameters.[26]

1.9 Summary of Animal and Human Studies on Accelerating OTM with LLLT

Taken together, animal and clinical studies illustrate the ambiguity that currently exists regarding the effect of LLLT on OTM. Accompanying this ambiguity of tooth movement effects, however, is the relatively clear message that photobiostimulation is not associated with any harmful effects on the periodontium and teeth (as assessed by radiographic and clinical assessments) and that it even that it has the potential to reduce pain during OTM.[15,55-57,59,65] Indeed, it has also been shown that LLLT may actually reduce tooth movement enough to decrease the amount of relapse

observed.[66] No root resorption greater than that normally observed during orthodontic treatment has been detected by cone beam computed tomography.[67] Radiographically, there was no evidence that LLLT had an adverse effect towards roots, alveolar bone, and periodontal tissues.[55,61] As well, no harmful response was seen clinically to the gingiva and mucosa following LLLT at the parameters studied.[68]

The ability to see both stimulatory and inhibitory results may be explained by the Arndt-Schultz Law (

Figure 3) which states that at low levels of stimulation the tissue response can be stimulatory and at higher stimulation we can observe inhibition in a biphasic dose response curve.[3]

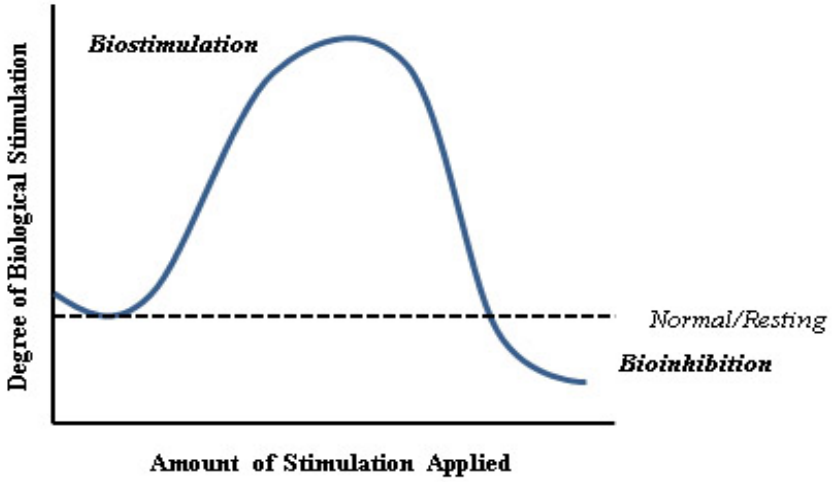

Figure 3. Ardnt-Schultz Law. Adapted from Reza et al. (2011)

The findings from both animal and human studies so far reveal conflicting results that showed both positive and negative effects of LLLT on the amount or rate of OTM. The extensive variability in the experimental parameters used between studies is a major contributor to the existing conflicting results and conclusions in the field. No available optical standards currently exist for the use of therapeutic lasers, making it confusing to make sense of the many different parameters that can be changed, such as wavelengths, power densities, application modes, and target tissues.[3] Even with the animal and clinical studies that did show positive effects of photobiostimulatory therapy, the change of tooth movement lies in the order of no more than 0.5 mm/month. Furthermore, the apparent benefit to OTM appeared to taper off with time, being

greatest at the initiation of OTM and subsequently decreasing. Given that the amount of tooth movement often ranges from 0-1mm per appointment it is still under debate whether the apparent increase of no more than about 0.5mm/month, although limited, has the potential to be clinically significant.[52]

The primary aim of this study is to test whether two different wattages of laser therapy with the same density, i.e., 500mW and 1000mW, will result in the same effects on OTM in the rat model. The secondary aim of the study is to assess for the presence of any negative tissue effects with the same treatment regime.

1.10 Hypothesis

The hypotheses, therefore, are 1) that orthodontically moved rat molar teeth exposed to the same energy density from a higher and lower wattage of LLLT show similar changes in the amount of movement and molecular profile changes in the periodontal cells and 2) that after LLLT exposure there are no negative side effects to the soft tissue.

1.11 Objectives

I. To assay differences in tooth movement and molecular changes in the PDL and socket of teeth undergoing OTM.
    a. Measurements of tooth movement through PVS impressions
    b. Real time PCR of markers of late OTM at 14 days

II. To assess for tissue damage and epithelial architecture after 14 days histologically

1.12 Significance of Study

This current study has the potential to clarify the conflicting results seen in the animal literature published on LLLT and increased rates of OTM. This experiment aims to clarify one parameter involved in LLLT, which is energy density, by way of validating the Ardnt-Schultz Law in an attempt to determine an ideal dose for LLLT in accelerating OTM. This would be a promising finding in the field of orthodontics as we are continually searching for ways to shorten treatment time and decrease the negative side effects of lengthy orthodontic treatment, such as periodontal diseases, plaque accumulation, caries, cooperation issues, and root resorption.[35,65]

Chapter 2
Methods and Materials

2.1 Animals

Approval for animal use was obtained from the University Animal Care Committee (UACC) at the University of Toronto (approval no. 20010570). A series of pilot studies were conducted to determine the presence of possible risks to the rats, the feasibility of the protocol and the optimal duration of tooth movement (see Appendix 2). The final experimental protocol consisted of a total of 27 six week old male Wistar strain rats (150-175g) exposed to two different laser settings over a duration of 14 days. The rats were kept in cages of 2 (except for one cage of 1) housed in a 12-hr light/dark environment at a constant temperature (23°C) and allowed to acclimatize for one week before the experiment. Mashed food and water *ad libitum* were provided, with no chew toys to reduce the risk of appliance failure. The weights of all 27 rats were recorded at the initial time-point and every other day before irradiation up to the day 14 end-point (Table 1).

2.2 Orthodontic Tooth Movement

The sample sizes of similar rat studies of OTM in the literature ranged from n=12 [49] to n=30.[45] In the current study, twenty-seven rats were divided into three groups, a control group (n=8), and two experimental groups subjected to 500mW (n=10) and 1000mW (n=9) of LLLT infrared irradiation. These groups will henceforth be referred to as CT, EX-500 and EX-1000, respectively. The reasoning for the difference of animals in each group was due to the importance of maintaining the highest possible numbers in each experimental group due to previous failures in the pilot studies conducted and possible risk of loss of animal life (Appendix 2). It was decided that if any animal loss was to occur it would be beneficial to have the highest possible numbers in the experimental groups to compare to each other and since 500mW was the closest wattage to that previously reported successful in the literature it received the highest number.

All groups had an activated coil spring placed for 14 days from the molars to the incisors to induce mesial movement of the molar according to previous reports.[43,45,39,49] To allow unlimited access to the oral cavity during coil spring placement, the rats were placed under intraperitoneal general anesthesia (GA) with 90mg/kg Ketamine and 5mg/kg Xylazine. A force of 10g was provided by an activated 25g NiTi coil spring (GAC Dentsply) that was placed between the first molar and the incisors on the left side. A dynamometer was used to measure the amount of activation to achieve 10g of force (equivalent to 11mm islet to islet) and checked intra-orally to ensure adequate activation. To prevent coil dislodgment, the lower incisors were trimmed at the start and throughout the experimental period to relieve occlusion. Proper activation of coil was checked every other day. The coil was secured at the molar and incisor with stainless steel ligatures. The incisors were prepared with lateral grooves from the buccal to lingual surfaces on the distal surfaces of the incisors as close as possible to the marginal gingiva for placement of the stainless steel ligature to provide mechanical retention. The palatal surface of the molar was bonded with a self-etching primer (TransbondPlus Self-Etching Primer, 3M Unitek, USA) and flowable resin (Filtek Supreme Ultra Flowable, 3M Unitek, USA) over the stainless steel ligature. The incisors were etched with 35% phosphoric acid for 10 seconds, rinsed and primer (Assure, Reliance, Canada) and resin (Transbond XT 3M Unitek, USA) were placed to chemically secure the stainless steel tie (Figure 4).

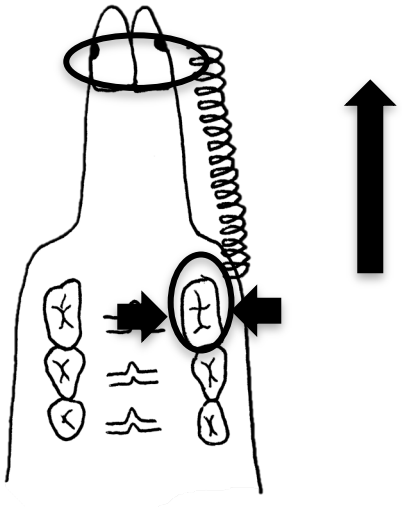

Figure 4 NiTi coil application in the rat model with stainless steel ties around incisors and left first molar

2.3 Laser Irradiation and Parameters

The Picasso Lite diode laser (AMD Lasers, Dentsply, GAC, USA) is a gallium-aluminum-arsenide (GaAlAs) diode laser with a wavelength of 810nm. It was used in a continuous mode at 500mW (lowest possible setting) and 1000mW with a 0.4mm diameter beam. This laser was chosen because it is one of the most readily available and widely used diode lasers in Canada (e.g., for soft tissue procedures), thus increasing the clinical applicability of this study.

The determination of the parameter of interest, i.e., energy density (J/cm^2 or Wxs/cm^2) was calculated based on four published studies that showed increased OTM in rats with LLLT (Appendix 1, Table 3).[39,43,45,49] Therefore, the exposure times of laser application to generate 19098.6 J/cm^2/tooth at 500mW and 1000mW was calculated to be 24 and 12 seconds each for the buccal and palatal gingiva, respectively (Appendix 1, Table 4).

2.4 Laser Exposure

The laser beam was applied through a 0.4mm diameter optical fiber. The fiber tip was held within 1mm from the gingiva without contact and moved in a circular motion over the root surfaces, first on the palatal and then on the buccal surfaces of the tooth, for a total of 24s and 48s for EX-1000 and EX-500, respectively. Control groups were comprised of rats subjected to the same orthodontic forces with no irradiation. The experiment was run for 14 days with seven LLLT sessions (every other day starting on the day of coil spring placement and activation). LLLT was performed under inhalational GA with isoflurane at 4% for induction and 2-2.5% for maintenance at 1L/min oxygen.

2.5 Measurement of Tooth Movement

A number of methods were tested for accuracy as to which method would allow for the best visualization and precision in measuring the gingiva between the first and second molar after OTM. Further details of these techniques are described in Appendix 3. A PVS impression was taken at appliance activation and end point to quantify tooth movement. In order to measure the amount of movement of the first molar, the PVS impression was viewed under a dissecting microscope. The midpoints of the gingival margin between the buccal and lingual surfaces of the first and second molars in the final impressions were marked and the distance was measured with

an electronic caliper (Figure 5). All measurements were performed under the dissecting microscope by two different observers at two different times (M.M. and another independent investigator blinded to the study).

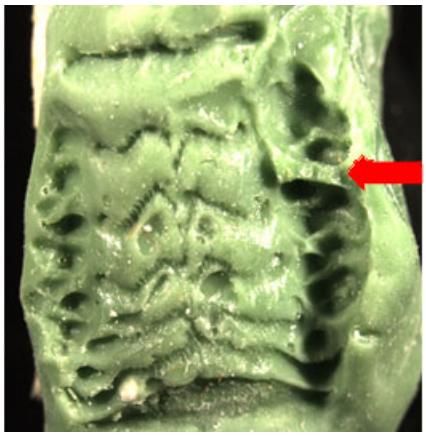

Figure 5 Image of final impression with direct visualization of marginal gingiva between first and second molars under the dissecting microscope (arrow)

2.6 Tissue Preparation

After 14 days, the rats were euthanized with carbon dioxide for a minimum of 5 minutes and absence of pulse was assessed. The coil spring was removed and the left first molar that had undergone OTM was extracted using a half Hollenback instrument. The extracted molar was immediately placed in an eppendorf tube and stored in liquid nitrogen for collection of PDL cells for RT-qPCR. After extraction, the palatal tissue was excised and stored in Bouin's fixative solution for histological analysis.

2.7 Gene Expression by RT-qPCR

RNA was extracted from the PDL cells of all the extracted left first molars in the three groups (EX-500 n=10, EX-1000 n=9 and CT n=8) using an RNeasy micro kit following the manufacturer's protocol (Purification of Total RNA from Animal and Human Tissues Protocol, Qiagen) and reverse transcribed using a Maxima Universal First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The cDNAs were subsequently used as templates for RT-qPCR using TaqMan Universal Master Mix II and TaqMan Gene Expression Assays for *GAPDH, RANKL,* and *MMP-13* (Applied Biosystems, Life Technologies, Canada). The RT-qPCR reactions for

each sample were run in 96-well plates in technical triplicates. The samples were denatured at 95°C for 15s, with an annealing temperature of 60°C, for 40 cycles at 1 minute.

2.8 Histology

Histological analysis was performed on CT (n=8), EX-500 (n=5) and EX-1000 (n=9) for qualitative assessment of tissue damage consequent to laser treatment. Three samples were initially taken for qualitative assessment of tissue damage consequent to laser treatment. Upon preliminary findings of obvious histological changes within the EX-1000 group, the sample sizes were increased in the CT and EX-1000 groups. Overall, histological analysis was performed on CT (n=8), EX-500 (n=5) and EX-1000 (n=9). The palatal tissue samples were fixed and dehydrated through an ethanol series and embedded in paraffin for processing according to Gong.[69] The samples were sectioned at 8µm thickness and processed for staining. The palatal tissue samples were fixed and dehydrated through an ethanol series and embedded in paraffin for processing according to Gong.[69] The samples were sectioned at 8µm thickness and processed for staining.

Slides were prepared for both H&E to assess the overall cellular characteristics and Masson's trichrome staining to assess production of keratin and changes to the connective tissue. After deparaffinization, rehydration and rinsing, the sections were stained in Weigert's iron hematoxylin working solution, washed and stained in Biebrich scarlet-acid fuschsin solution for 10 - 15 minutes, washed and further stained with phosphomolybdic-phospho-tungstic acid solution. The slides were transferred directly to aniline blue solution, washed and differentiated in 1% acetic acid solution, dehydrated, cleared, and mounted. The number and morphology of both epithelial and fibroblast cells were qualitatively compared between the LLLT groups and control using Masson's trichrome stain. To quantify the fibroblasts in the different groups, one section was taken from three different animals in each group. Images of the mesenchymal areas of the gingiva were captured at 100X magnification. The number of fibroblast cells within a 100µm square area close to and underlying the epithelial layer were counted. All of the samples in EX-1000 and five of the EX-500 group were analyzed for epithelial changes to determine the frequency and degree of changes present using both H&E and Masson's trichrome stain in comparison to control.

2.9 Statistics

Values of the amount of tooth movement were calculated and presented as the mean ± standard error (SE) for each group. Intergroup comparisons for OTM and gene expression were performed with a Mann-Whitney U-test (since the data was not normally distributed) and a value of $P<0.05$ was considered to indicate a statistically significant difference. For measurement of tooth movement, an interclass correlation (ICC) was run to determine the level of agreement between observers. Before final data analysis, one outlier in the EX-1000 group and one sample in the CT group (that demonstrated dramatically increased gene expression of both MMP-13 and RANKL) were removed after validation using the modified Thompson tau test.

Chapter 3
Results

3.1 Animals

There was no statistical difference in the starting average weights of all 27 rats (P=0.80), with CT= 211.88±10.2g, EX-500= 221.40±10.1g and EX-1000= 212.33±9.21g (Table 1). All animals exhibited weight increase over the course of the study, with no statistical significance between the three groups (P=0.609) (Table 1). No animals required premature euthanasia due to excessive weight loss and all rats maintained their appliances for the duration of the 14 day experiment and were grooming normally.

Table 1 Mean initial (Day 1) and final (Day 14) weights and overall total weight gain (mean weight gain) (± standard deviations)

	Mean Initial Weight (±SD) (g)	**Mean Final Weight (±SD) (g)**	**Mean Weight Gain (±SD) (g)**
CT	211.9 (±10.2)	306.0 (±12.6)	94.1 (±10.1)
EX-1000	212.3 (±9.2)	312.0 (±18.9)	99.7 (±12.7)
EX-500	221.4 (±10.1)	317.0 (±16.1)	95.6 (±12.4)

3.2 Tooth Movement Changes in Experimental and Control Groups

Excellent agreement was found between the examiners in the measurements of the distance between the first and second molars (ICC = 0.93).

After 14 days, the average OTM was 0.639±0.057, 0.745±0.051 and 0.753±0.057mm for CT, EX-1000 and EX-500 groups, respectively (Table 2). One outlying sample in the EX-1000 group was removed from the data analysis (2.06mm versus the group average of 0.745mm). A statistically significant increase was observed for the EX-500 group compared to CT (P=0.041), however, there was no statistical difference between EX-1000 and CT. The difference in the

amount of tooth movement between both laser groups was not statistically significant (P=0.329) (Table 2 and Figure 6).

Table 2 Average amounts of tooth movement (± standard errors)

	Sample Size (N)	**Average OTM (± SE) (mm)**
CT	8	0.639(± 0.057)
EX-1000	8	0.745(± 0.051)
EX-500	10	0.753(± 0.057)

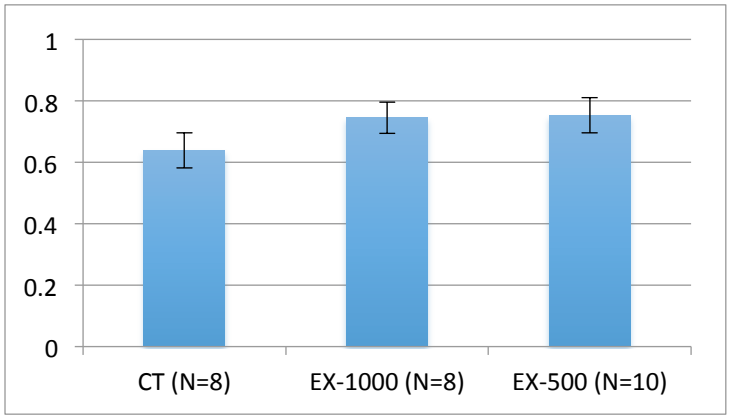

Figure 6 Graph of average tooth movements in mm (± standard errors)

3.3 Gene Expression with RT-qPCR

The expression of *MMP-13* and *RANKL* was up-regulated in both experimental groups compared to CT group. The expression levels of *MMP-13* and *RANKL* were significantly higher in the EX-1000 compared to CT with the gene expression [3.79±0.81 (P<0.004) and 6.26±0.76 (P<0.001) fold, respectively]. Similarly, there was also a statistically significant increase in expression levels in the EX-500 group for both *MMP-13* and *RANKL* [5.24±0.73 (P<0.005) and 3.36±0.72 fold (P<0.008), respectively] (Figure 7). The change in expression levels of *MMP-13* was highest in the EX-500 group, with an average difference of 1.45 fold between treatment groups. Conversely, the EX-1000 group exhibited a greater change in *RANKL* expression levels, with an average difference of 2.91 fold between treatment groups. No statistically significant differences were found between the two treatment groups for both genes (P=0.568 for *MMP-13* and P=0.369

for *RANKL*). One sample from the control group exhibited very high levels of *MMP-13* and *RANKL* for unknown reasons and was excluded from analysis (Figure 7).

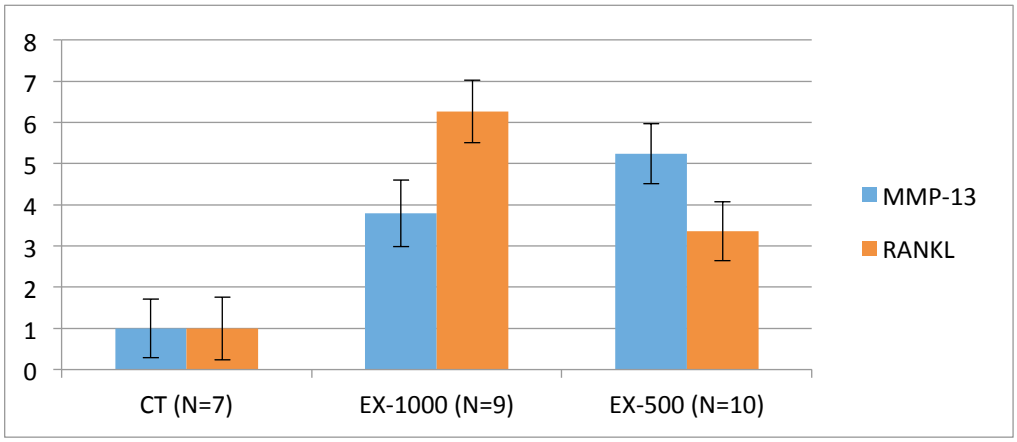

Figure 7 Fold gene expression for MMP-13 and RANKL (± standard errors)

3.4 Histological Changes in Gingiva

Although no tissue necrosis was found, changes in both the epithelia and the underlying connective tissues in the histological sections of palatal gingiva adjacent to the molars subject to laser treatment were clearly evident in the EX-1000 group. 5 out of the 9 samples (56%) of the EX-1000 rats examined exhibited thickening of the epithelial and keratinized layers (compare D with E and F, Figure 8, all taken at same magnification). A greater number of round and small epithelial cells were present in the epithelial layers, more towards the basal half of the gingiva (arrows in L, Figure 8). That these changes were suggestive of hyperplasia and increased proliferation of basal layer cells in the EX-1000 groups was corroborated by the presence of elongated rete ridges (arrows in F, Figure 8). In spite of the overall increased thickness of the epithelial layers in the EX-1000 groups, the presence of the typical layers of parakeratinized tissues, i.e., basal, prickle cell, granular, and keratinized layers were still evident. Compared to the CT group, no major histological changes were observed in the EX-500 group, although, in some samples, there was increased thickness of the keratin layer.

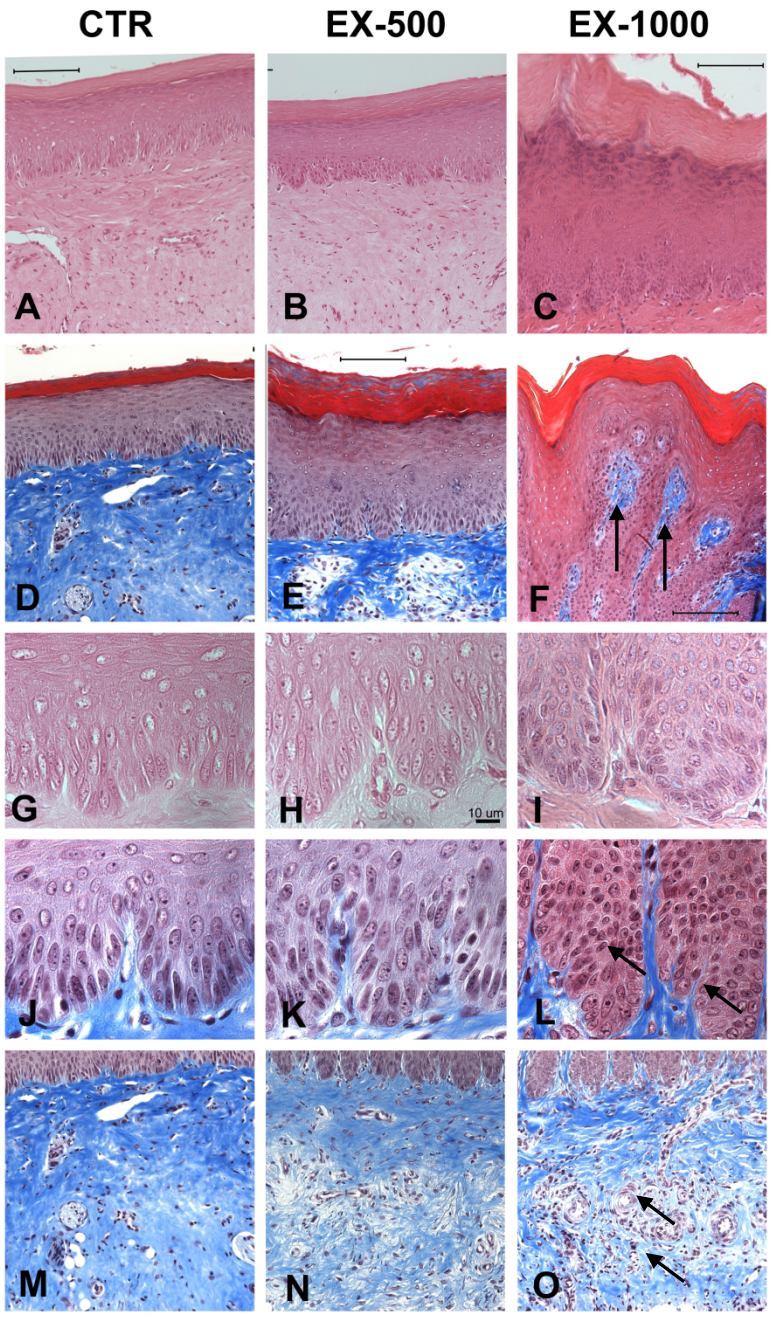

Figure 8 Histological images of palatal gingival tissue at under the light microscope. A, B, C, D, E, F, M, N, O are at 20X with scale to 100um. G, H, I, J, K, L are at 100X oil immersion with scale to 10um. A – Control H&E stain at 20X, B – EX-500 H&E stain at 20X, C- EX-1000 H&E stain at 20X, D – Control Masson's Trichrome stain of epithelium at 20X, E – EX-500 Masson's Trichrome stain of epithelium at 20X, F – EX-1000 Masson's Trichrome stain of epithelium at 20X, G –Control H&E stain of basal layer at 100X, H – EX-500 H&E stain of basal layer at 100X, I – EX-1000 H&E stain of basal layer at 100X, J – Control Masson's Trichrome stain of basal layer at 100X, K – EX-500 Masson's Trichrome stain of basal layer at 100X, L – EX-1000 Masson's Trichrome stain of basal layer at 100X, M – Control Masson's Trichrome stain of connective tissue at 20X, N - EX-500 Masson's Trichrome stain of connective tissue at 20X, O - EX-1000 Masson's Trichrome stain of connective tissue at 20X

The connective tissue demonstrated changes in both laser groups compared to control. A greater number of fibroblasts were observed in the laser treated groups compared to the CT group (compare the presence of numerous dark fibroblastic cells, some arrowed, between M, N, O in Figure 8 and Figure 9). The means of the three measurements were compared between the groups and both laser groups were found to have a statistically significant increase in the number of fibroblasts compared to controls ($P<0.05$).

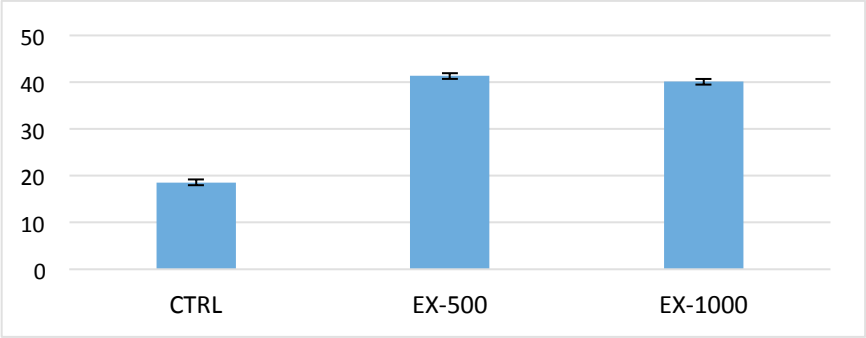

Figure 9 Number of fibroblasts, mean of cell count at 100X magnification oil immersion (± standard deviations)

3.5 Summary

The average difference in the amount of tooth movement increase was 0.106mm with EX-1000 and 0.114mm with EX-500 groups compared with the CT group after 14 days. There was a statistically significant increase in tooth movement between the CT and EX-500 ($P<0.05$) group with no statistically significant difference between the two LLLT groups. At the molecular level, there was a statistically significant increase in gene expression for both *MMP-13* and *RANKL* in both laser treated groups compared to control, with no statistically significant difference between the experimental groups. At the histological level, no obvious histological changes in the epithelial layer were observed in the EX-500 group but epithelial changes were observed in about half of the samples in the EX-1000 group. The changes observed included increased epithelial thickness, elongated rete ridges, changes in size of cells in the basal layer, and an increase in the number of fibroblasts.

Chapter 4
Discussion

The study set out with the major goal of determining if LLLT, delivered at the same energy density but at two different settings, was capable of demonstrating the same stimulatory effect on OTM. The results not only answered that specific question but also raised several interesting ones that will be discussed in the following sections.

4.1 Molecular and Histological Changes

Of all the changes that were observed, the most striking were those at the molecular and histological levels that did not appear, however, to be manifested to the same degree by changes in the amount of tooth movement. The assay methods for each individual parameter, i.e., tooth movement, molecular changes in the PDL and histological changes in the adjacent gingiva, vary significantly in their degree of sensitivity. The ability to detect changes at the molecular level, even in the presence of small amounts of starting material, i.e., the PDL tissues, has been made possible with the use of the powerful and sensitive assay methodology of RT-qPCR. The use of that technology combined with our study design of assaying the molecular changes in *triplicates* of each tooth booster our confidence that the molecular changes were highly representative of the changes in the PDL of teeth subject to laser treatment and force application.

The expression of *RANKL* and *MMP-13* was assayed in this current study based on their documented activities in bone remodeling, a key event during OTM. Both genes play a role in osteoclast activity on the compression side of the molar during OTM[70]; *RANKL* has been reported to be released by both fibroblasts and osteoblasts[39] and MMPs play a pivotal role in bone degradation. Specifically, *MMP-13* has been shown to play a direct role in bone matrix dissolution[71] and *MMP-9* was reported to be one of the major proteases released by osteoclasts during invasive activity.[43] Our finding of a statistically significant increase in *RANKL* expression after LLLT corroborate those found by Fujita et al. who demonstrated a significant increase in *RANKL* presence in light emitting diode groups, albeit analyzed via immunohistochemistry, a less sensitive technique compared to RT-qPCR.[39] The fact that no statistical differences in gene expression in both *RANKL* and *MMP-13* were observed between the laser-exposed groups helps

validate the similarity of bone remodeling occurring for both wattages used and is in agreement with the comparable amounts of tooth movements observed in the laser groups. Overall, therefore, the current study showed clearly that the use of LLLT using the methodology and parameters as outlined in the current study was capable of stimulating two specific molecules involved in both osteoblast and osteoclast function. However, this stimulation may not necessarily translate into a clinically useful difference in the amount of tooth movement.

It was important to determine, at the cellular level, if there was any tissue ablation that occurred and if these risks would outweigh the observed increase in tooth movement. Histologically, the changes observed in 56% of the EX-1000 group were quite dramatic. There were early histological signs of dysplasia, many of which were reminiscent of the reactions to laser therapy after the creation of a surgical wound. That is, healthy tissues after being exposed to high intensity laser (1000mw) exhibited the presence of mitotic figures within the stratum germinativum and long and irregular projections of connective tissue into the underlying epithelial surface.[72] Similarly, histological changes observed after wound healing treatment with LLLT biostimulation included acceleration in the maturation of keratinocytes, increased proliferation of myofibroblasts and fibroblasts, increased collagen deposition and hyper- and parakeratosis in the epithelium.[73-76] The finding of increased number of fibroblasts in the connective tissue in the current study is in agreement with the literature[37,38] and is especially pertinent to the study of orthodontic relapse. However, it is difficult to determine whether an increased number of fibroblasts could lead to accelerated soft tissue remodeling or whether the increased cellularity would create resistance to tooth movement.[77-79] Laser therapy is used extensively in dentistry and, therefore, the implications of the soft tissue histological changes after laser exposure are clinically significant. The recommended wattage in the literature and commercial product literature is 500mW for the treatment of aphthous ulcers for pain reduction and healing.[80] For soft tissue ablation procedures, usually a one-time procedure, 800mW is recommended by Biolase (EZlase, USA). The possibility exists therefore, that at that particular wattage (which is just slightly lower than the 1000mW in this current study), dysplastic changes may occur. Further studies are warranted to assess the extent of dysplastic changes after the exposure to laser use and if so, whether these changes are a permanent or transient result of LLLT. There has been no documentation of the gingival changes that accompany teeth undergoing LLLT with orthodontic forces and our findings do show that higher wattages may

have a negative effect on epithelium if used for a prolonged period, as would be done for LLLT. This may be an indication for a recommendation against the use of 1000mW for procedures like LLLT until further investigations have assessed whether this change in epithelial morphology can return to normal after removal of photobiostimulation.

4.2 Observed OTM Compared to the Literature

Several different methodologies for measuring OTM have been reported. The use of intraoral measurements[35] was decided against in the current study due to the difficulty of visualizing the first molar and placement of the calipers in a stable position. Several studies used a contact-type three-dimensional measurement apparatus.[39,43,49] One example of this includes the use of the orthodontic software, designed primarily for patients (OrthoCAD, AlignTechnology, USA) that only measures to 0.1mm and therefore does not afford enough precision for our rodent study. In this current study, taking measurements off the PVS impressions allowed direct measurement of the marginal gingiva and more accurate assessment of bodily movement. While there was a statistically significant difference in tooth movements between CT and EX-500, the average OTM changes in all groups was less than 1mm after 14 days of laser exposure. The data, therefore, clearly highlight the inherent lack of precision in the ability to measure the amount of tooth movement, especially in a rodent model. One way to overcome the small changes would be to increase the sample size of the study. Another would have been to use computed tomography (CT) for more precise quantitation of tooth movement as more recent studies have used an *in vivo* micro-CT for the assessment of OTM.[45] Both strategies, however, would substantially increase the cost and labor intensity of such studies.

Direct comparison of the amount of tooth movement observed in this study and others in the literature is difficult, as only fold differences were reported in other studies. The fold differences in the current study were 1.17 for 1000mW (0.745mm/0.639mm) and 1.18 for 500mW (0.753mm/0.639mm), values that were slightly lower than the 1.26 fold at day 14 by Yoshida et al.[45] and 1.3 fold at day 12 by Kawasaki & Shimizu.[49] Comparisons of the actual tooth movements in the study by Yoshida et al., as inferred from graphical data, showed values of 0.33mm and 0.26mm in laser and control groups, respectively, as compared to values of approximately 0.745mm, 0.753mm and 0.639mm in the EX-1000, EX-500 and control groups, respectively, at 14 days.[45] The reason for the differences between the overall tooth movements

between the current and published studies are not easily discerned as the same coil spring material and force application was applied. It might be possible, since minimal activation was necessary for the force of 10g, that the coils in the other studies may have lost their activation since no mention was made by the authors regarding reactivating or monitoring of spring activity.

4.3 Energy Density and the Ardnt-Schultz Law

The initial selection of the parameter for analysis during LLLT use was difficult because of the numerous options available. The final decision to investigate energy density in the current study was based on practical reasons and clinical applicability. A commercially available diode laser that is available in an orthodontist's office was chosen; however, as 500mW is the lowest wattage settings in these lasers, a wattage that is higher than previously reported in other studies, a different parameter had to be kept constant. The energy density chosen was based on four studies showing successful acceleration of OTM.[39,43,45,49] While the energy density at every session could be maintained, the total energy dose was not the same due to varying experimental durations and differing dosage schedules (every other day in current study compared to every day for the first four days and then on the 7th day afterwards in other studies).[39,43,45,49] The irradiation schedule was made more consistent in the current study in an attempt to provide a more constant rate of tooth movement. Due to the ethical considerations of animal use, the use of inhalational anesthesia was only allowed every other day to ensure adequate recovery between exposures. Compared to other studies, there was no direct physical contact with the laser to the gingiva to avoid any possible negative side effects of tissue ablation. Laser was also applied differently in the current study by moving the laser in a constant circular motion within 1mm of the buccal and lingual gingival surfaces, with the hope that this would allow more root surface stimulation and minimal tissue ablation in comparison to studies that used several points of laser application over the buccal and lingual root surfaces.

The current study revealed a trend towards an increased rate of OTM. It is possible that a larger sample size would result in a finding of a statistically significant difference in the amount of tooth movement for both the laser groups compared to the control group. Even if a statistical significance is found it is still difficult to determine if this increased rate is clinically significant. The similarity of observed OTM for both laser groups supports the Ardnt-Schultz Law for the

parameter of energy density that states that two different wattages used for appropriate times will show the same level of stimulation. However, since the overall tooth movement in the current study was higher than those previously reported in other similar studies, it remains difficult to say where the chosen energy density falls on the curve and if other energy densities may be more stimulating. In order to understand the true validity of the Ardnt-Schultz Law a dose-response curve relationship has to be established.

4.4 Conclusion

Of the currently available systematic reviews that addressed the question of whether LLLT accelerates OTM[26,51,61,68], many contain bias that precludes a clear answer and lacks the presentation of a clear clinical protocol that could be adopted for future studies. The findings of this current study add new information to the current literature in its presentation of the molecular stimulatory effects at a given energy density, regardless of wattage, and the possible histological changes at a higher wattage. Before laser treatment modality can truly be useful clinically to accelerate OTM, future studies should focus on the elucidation of the possible biphasic response of various energy densities while keeping all other parameters constant. This will help the field of orthodontics in its continual search for non-invasive ways to shorten treatment time and decrease the negative side effects of lengthy orthodontic treatment, such as periodontal diseases, plaque accumulation, caries, cooperation issues, and root resorption.[35,65] As well, the use of 1000mW for photobiostimulation is not recommended due to the potential for dysplastic changes that may arise until further investigation to understand the potential harm is conducted.

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Appendices

Appendix 1 Laser Irradiation and Parameters

Table 3 Successful animal LLLT protocols in the literature

Protocol	**Yoshida[45]**	**Fujita[39] and Yamaguchi[43]**
Beam diameter (d)	0.6mm	0.6mm
Beam radius (r)	0.3mm	0.3mm
Beam area (πr^2)	0.283 mm^2 0.00282cm^2	0.283 mm^2 0.00282cm^2
Wattage (W)	100mW = 0.1W	100mW = 0.1W
Time per point (s)	135s	180s
Energy (J)	13.5J per point x 4 points = 54J/tooth	18J per point x 3 points =54J/tooth
Energy density (J/cm^2)	13.5J/point/0.00282cm^2 =4774.6 J/cm^2/point 54J/tooth/0.00282cm^2 **=19098.6 J/ cm^2/tooth**	18J/point/0.00282cm^2 =6366.2 J/cm^2/point 54J/tooth/0.00282cm^2 **=19098.6 J/ cm^2/tooth**

By calculating the total energy density per tooth the exposure times of laser application for the two wattages could be calculated (Table 4 **Calculation for exposure times at two wattages**).

Table 4 Calculation for exposure times at two wattages

Group	**1000mW**	**500mW**
Energy density/tooth (J/cm^2)	19098.6 J/cm^2/tooth	19098.6 J/cm^2/tooth
Beam diameter (d)	0.4mm	0.4mm
Beam radius (r)	0.2mm	0.2mm
Beam area (πr^2)	0.126mm^2 0.00216cm^2	0.126mm^2 0.00216cm^2

Energy/tooth (J)	19098.6 J/cm^2 x 0.00216cm^2 = 24J	19098.6 J/cm^2 x 0.00216cm^2 = 24J
Total time/tooth (s)	24J/1.0W =24s	24J/0.5W =48s
Time per buccal and lingual (s)	24s/2 =**12s each**	48s/2 =**24s each**

Appendix 2 Pilot Studies

I. First Pilot Study

Twelve rats (two groups of n=6 for control and LLLT groups) over a period of 21 days at the highest wattage were tested for the most difficult conditions for the animals. According to published works, an activated coil spring with a force of 10g from the molars to the incisors was used to induce mesial movement of the molar.[39,43,45] One disadvantage with this methodology was the confounding variable of iatrogenic inflammation in the gingival areas if a stainless steel ligature was inserted through the contact of the first and second molar. To eliminate this concern, bonded light-cured resin (Transbond XT, 3M Unitek, USA) was used to chemically secure the coil springs to the molars. The molars and incisors were etched with 35% phosphoric acid before preparation with self-etching primer (Transbond Plus Self-Etching Primer, 3M Unitek, USA) and light-cured resin. One major difficulty encountered with this protocol was the number of bonding failures at the molars, followed by problems with replacement of wires with adequate activation. Problems were encountered with the intraoral use of a dynamometer that did not allow for adequate readings due to the tooth movement of the rat with application of force, leading to coils being under-activated or dislodged, a predetermined euthanasia end point.

During surgery, the rats were anesthetized under GA with isoflurane at 4% for induction and 2-2.5% for maintenance at 1L/min oxygen. Difficulty was encountered during placement of the activated coiled spring due to the presence of the nose cone that was used for delivery of the GA coil spring placement. Several rats awoke during coil spring placement at the molars making

isolation extremely difficult and the coil placement very time consuming. An accurate assessment of OTM could not be established but it was decided to continue the experiment to the 21 day end point to assess whether any gingival damage could be discerned clinically. While the gingival tissue at the end of the experiment appeared unchanged after laser irradiation the results of this pilot experiment were unsuccessful. There was no OTM observed in the control group due to isolation difficulty during bonding, bond failures at the molar, and anesthesia. It was agreed that another pilot study was required with modifications to the methodology to improve coil spring retention.

II. Second Pilot Study

The goal of the second pilot experiment was to determine if the coil springs could be maintained at the molars for 21 days. Again, 12 rats (n=6 for control and LLLT groups) were tested for 21 days with the 1000mW setting. To insure complete anesthesia for appliance placement a request was sent to Health Canada and was granted for the use of Ketamine (Authorization 35909.12.14 Expiry: 2015-12-23). The rats were placed under intraperitoneal (IP) GA with 90mg/kg Ketamine and 5mg/kg Xylazine. This allowed for unlimited access to the oral cavity during coil spring placement. The coil spring was measured on the benchtop to establish the distance required for 10g of activation with a dynamometer. The coil was then measured with a compass locked to the desired distance once placed intraorally to ensure appropriate activation. Surprisingly, coil spring failures were occurring at the incisors, where the methodology had proven successful previously. It was determined that the convex surface of the incisors placed the resin bonding of the stainless steel tie at risk of debonding upon mastication. It was also noted after two weeks that the coils were losing activation and replacement was attempted but, again, due to the nose cone, improved activation could not be obtained and would require an additional IP injection of Ketamine and Xylazine. Due to this reason the protocol was changed from 21 days to 14 days. This timepoint would still allow for characterization of the accelerated phase of OTM. It was determined that the longest timepoint (14 days) would be analyzed before commencing a second experiment for the shorter timepoint to avoid wasting animal life if the results were not found to be significant. To improve the mechanical retention of the stainless steel ligature the incisors were prepared with lateral grooves from the buccal to lingual surfaces on the distal surfaces of the incisors as close as possible to the marginal gingiva for placement of the stainless steel ligature.

Appendix 3 Measurement of Orthodontic Tooth Movement

A PVS impression was taken at the appliance activation and end points to quantify tooth movement. A number of techniques were attempted to determine which would provide the best accuracy for measuring bodily movement of the first molar.

Photography was utilized from a fixed distance with angled lighting to highlight the landmarks of the casts with a ruler placed at the occlusal table for scale. The photographs were then imported into ImagePro Software (ImagePro 2.0) to measure the distance from the distal marginal ridge of the first molar to the distal marginal ridge of the second molar with the values of the final cast subtracted from the initial cast. This method did not allow for accurate comparison of initial and final casts due to the difficulty of aligning the occlusal plane.

To better compare the initial and final casts it was decided to analyze them in 3-dimensions (3D). The first method assessed for 3D analysis was to scan the casts with micro-CT (Scanco Medical AG, Switzerland, model μCT40). To gain additional heterogeneity of the stone cast, one model was coated in gold. This methodology is only useful for *in* vivo samples as the density differences between bone, dentin, and pulpal tissues allows for improved visualization of spaces. It was unsuccessful with the stone models.

Another potential technology for 3D assessment was the iTero intraoral scanner (AlignTechnology Inc., USA). The iTero scanner has been published to be accurate to within 10μm and is used in the field of prosthodontics for single tooth restorations and is now being used in the orthodontic field.[81] It is commonly used for space analysis through the orthodontic software OrthoCAD to assess excess or deficient spacing in the dental arch for orthodontic treatment planning. Due to legal implications of measurements on an animal and the software having to place the scan on an orthodontic model base the software was unable to accommodate the requirements for this study. As well, the measuring tool in the software could only measure to 0.1mm which was not sensitive enough for the small differences in OTM observed.

The PVS impressions were assessed for their ability to be measured directly as they give direct visualization of the marginal gingiva between the first and second molars. The final impressions were measured and marked to show the midpoint of the gingiva between the buccal and lingual surfaces created after tooth movement. Measurements were performed with digital calipers under

the dissecting microscope by two different observers at two different times. It was decided that direct measurement from the PVS impressions provided the most accurate measurement for bodily movement of the first molar.

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