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Analysis of the biological effects of therapeutic ultrasound on orthodontically induced tooth root resorption repair

by

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Dedication

To my parents Matrafa and Mohammad, lovely wife Sarah, brothers (Ibrahim, Turki, Majed, Hassan, Hussain, Ali, Ahmad, Hadi, Abdullah and Jafar), my sister Sarah and my sweet daughter Albatoul.

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Abstract

The aim of this thesis was to investigate the biological effects of LIPUS on orthodontically induced inflammatory resorption (OIIRR) in *in vivo* and *in vitro* models. The in vitro samples were obtained from healthy human premolars and cultured for 5-days or 24-hours with application of LIPUS at different doses. The in vivo, we tested 10 beagle dogs where orthodontic movement was carried out for four weeks with a continuous force of 1 N/side; using a split mouth model. After 4-weeks, mandibles were resected into blocks involving the fourth premolar and its periodontal tissue. The 4^{th} premolars were evaluated using micro-CT, histologically and immunohistochemically. In both models, LIPUS was generated with repetition rate of 1 KHz. Each pulse has a square envelop with duration of 200 microseconds and a carrier frequency of 1.5 MHz and 30mW/cm^2 intensity. Daily application of LIPUS on TSOC for 5-days did not have any effect on the predentin thickness layer, and had an adverse effect on the odontoblast cell count. A one-time application of LIPUS in a 5-day culture of TSOC increased the predentin thickness in all groups. Also it increased the odontoblast cell count in the 5, 10 and 15 minutes application groups. LIPUS application for 10 minutes upregulated the expression of collagen-I and DMP-1 in the short term (24 h), where 5-minutes application upregulated the expression of collagen-I only. LIPUS did not affect the rate of orthodontic tooth movement and had a trend of increasing it with increased population of the osteoclasts attached to the alveolar bone in the PDL. LIPUS significantly reduced the number of OIIRR initiation areas by 71%, reduced its total volume by 68%, and reduced its volume relative to

the affected root total volume by 70%. LIPUS induced the formation of precemntum layer, thicker cementum and reparative cellular cementum.

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ALP: alkaline phosphatase.

ATP: Adenosine 5'-triphosphate.

B: Buccal.

BMP: Bone morphogenic protein.

Ca: Calcium.

CD40: Cluster of differentiation molecule, (costimulatory protein found on antigen presenting cells).

COL-I: Collagen type-I.

CTL: Control.

D: Distal.

DMEM: Dulbecco's Modified Eagle Medium.

DMP1: Dentin matrix protein-1.

DPP: Dentin Phospophyren.

DPSCs: Dental pulp stem cells.

DSP: Dentin Sialoprotein.

DSPP: Dentin Sialophosphoprotein.

EARR: External apical root resorption.

EDTA: Ethylenediaminetetraacetic acid.

F: Fluoride.

FEA: finite element analysis.

H & E: Hematoxylin and Eosin.

HDE: Human dentin extract.

hsp: Heat shock protein.

Hz: Hertz

ICC: Intraclass correlation coefficient.

IL- : Interleukin.

L: Lingual.

LIPUS: Low Intensity Pulsed Ultrasound.

M: Mesial.

M-CSF: Macrophage colony stimulating factor.

OIIRR: Orthodontically induced inflammatory root resorption.

OPG: Osteoprotegrin.

OPN: Osteopontin.

P: Phosphorous.

PBS: Phosphate-buffered saline.

PDL: Periodontal ligament.

PTH: Parathyroid hormone.

RANKL: Receptor activator nuclear-κ ligand.

RL: Resorption lacunae.

RT: Room temperature.

RT-PCR: Real time polymerase chain reaction.

TGF-β1: Transforming growth factor ß1.

TNF: Tumor necrosis factor.

TNF-R: Tumor necrosis receptor family.

TRAP: Tartrate resistant acid phosphatase.

TRR: Tooth-root resorption.

VEGF: Vascular endothelial growth factor.

W: Watt.

μ-CT: Micro-computed tomography.

CHAPTER 1

Introduction

1.1 Statement of the problem:

Tooth-root resorption (TRR) is one of the adverse outcomes of dental trauma, orthodontic tooth movement and dental replantation/transplantation. Also, TRR occurrence can be idiopathic in nature. One variation of the TRR is the orthodontically induced inflammatory root resorption OIIRR, which is considered the second most common side effect of orthodontic treatment after white spot lesions.[1,2] It is the consequence of a sterile inflammatory process that is extremely complex and multifactorial and happens in the periodontal ligament area when orthodontic force moves the root inside the PDL space and causes compression of the PDL tissue and ultimately occlusion of the blood vessels. Although, the outcome is frequently similar to other forms of root resorption, orthodontic root resorption is distinct from the other types of root resorption. It has been shown that there is a strong relationship between orthodontic tooth movement and external root resorption with evident individual variation in its severity.[6-9] The present treatment protocol for teeth diagnosed with severe OIIRR involves postponing the treatment, [10-12] and compromising treatment objectives/plans.[13] In order for the clinician to complete orthodontic treatment as planned in such conditions, there is a need for a non-invasive technique to minimize, prevent or treat OIIRR in high risk patients. In addition to compromising the crown-root ratio, root resorption has led to malpractice litigation against orthodontists.[15-17] A new non-invasive technique to prevent OIIRR would minimize the likelihood of such litigation when used early enough in treatment in high-risk cases.

Despite multiple investigations, the underlying mechanism of OIIRR and its predisposing factors, it is still unknown and considered to be a multifactorial process with many predisposing factors. Although the molecular mechanism of OIIRR remains unknown, [3] potential treatment modalities have been explored. Unfortunately most of these treatments were either invasive or not clinically applicable. Low Intensity Pulsed Ultrasound (LIPUS) is a non-invasive treatment modality that has been found to enhance healing of various types of traumatized connective tissues and to stimulate dental tissue formation. Previous research examining the effect of LIPUS mainly focused on independent tissue lines and cell culture. Few studies investigated the possibility of LIPUS as a treatment of OIIRR after simple (tipping) tooth movement.[4,5] There is a need to investigate the possible effect of LIPUS on prevention of OIIRR after complex tooth movement, for example bodily movement. Also, these previous studies [4,5] were not able address the mechanism of LIPUS and its potential role in OIIRR prevention and/or repair. LIPUS effects were studied on cell lines of the dentin-pulp complex. However, there is a complex interaction between pulpal cells and dentin that is dependent on normal architecture of this complex. It is possible that LIPUS may have different effects on the tissue as a whole without being identified with studying single cell lines.

From the previously investigated potential OIIRR treatment approaches, none is capable of being used simply in a clinical situation on humans except LIPUS. A clinical trial was conducted on a small sample of female patients and the results were indicative of the reparative potential of LIPUS [5]. It was assumed

that LIPUS had a stimulatory effect on cementoblasts and odontoblasts where it induced cementum and dentin matrices, which made the tooth less vulnerable to OIIRR. The effects of LIPUS treatment in stimulating tissue healing are reported to be dose dependent [18]. Assuming LIUPS has a positive effect in reducing OIIRR, this effect is not known it was reparative, preventive or both. The effect of LIPUS on orthodontic tooth movement, dental pulp, periodontal ligament as well alveolar bone during treatment of root resorption still required additional research.

Although *in vitro* models are useful in evaluating dental pulp and periodontal ligament cellular tissue response to LIPUS, extreme caution must be exercised in the interpretation of these findings in patient care. An *in vivo* study on an animal model is also required to analyze the hard and soft tissue changes and clinical outcome due to LIPUS, which will form a bridging, step to human study. The different phylogenic affinities among lower animals such as mice, rats, and rabbits, as compared with humans, makes extrapolation of research results from these animal models questionable to test the treatment outcome of some therapeutic agents or techniques. It is well known that the growth pattern of cementum in lower animals involves continuous eruption, with cementum being formed throughout their lifetimes. Unfortunately the use of higher animals such as monkeys in studying root resorption must be performed with an adequate sample size, but this is hard to achieve because of the high cost of these animals. Beagle dog is considered to be a good model with lower cost. It was used in previous studies for testing periodontal treatments and root resorption severity; hence it was our experimental model of choice.

1.2 Thesis objectives:

1. To determine if there is a dose dependent stimulatory effect of LIPUS on odontoblasts and the dentine-pulp complex *in vitro*.
2. To determine whether the stimulatory effect of LIPUS is mediated by general growth factors or an effect mediated by non-collagenous proteins.
3. To study the effect of LIPUS on the tumor necrosis factor-receptor family mediated odontoclastic differentiation mechanism in the human tooth pulp cell population in an *in vitro* model.
4. To evaluate the effect of LIPUS on the orthodontically induced root resorption, dentin and cementum matrix production, on alveolar bone remodeling and potential interaction between bone remodeling and OIIRR healing processes in a Beagle Dog animal model during tooth movement.

1.3 Thesis hypotheses:

First hypothesis:

LIPUS has a stimulatory effect on the excretion of dentin matrix by odontoblasts and its mineralization process.

Second hypothesis:

LIPUS effect on the excretion ability of odontoblasts is mediated by the transforming growth factor ß1.

Third hypothesis:

LIPUS has an effect on the receptor activator nuclear-κ ligand (RANKL)/ osteoprotegrin (OPG) based odontoclastic induction mechanism in the human dental pulp whose differentially are responsible for mineralized matrix resorption.

Fourth hypothesis:

LIPUS treatment for 20 minutes per day from the buccal surface during orthodontic bodily tooth movement will be effective in preventing and repairing OIIRR *in vivo* without adverse effects on the dentin pulp complex and tooth movement.

1.4 Study Design and Approach:

We divided this investigation into two major studies; the first was an *in vitro* study that investigated LIPUS effects on the dentin pulp complex. The second was an *in vivo* study where we investigated the effect of LIPUS on both PDL and dentin pulp complex during orthodontic tooth movement and associated OIIRR in a beagle dog model.

We tested the effect of LIPUS on the dentin-pulp complex by conducting an *in vitro* study on a human tooth slice organ culture. By applying LIPUS to this organ culture model we investigated the response of the dentin pulp complex cell population to different durations of LIPUS application. The response was evaluated histologically by measuring the predentin layer thickness, which is the non-mineralized organic matrix front of the hard tissue of the tooth (Dentin) secreted by the odontoblasts. This layer is expected to give an indication of the effect of LIPUS on the excretory activity of the odontoblasts and testing the first hypothesis. Moreover the odontoblastic cell layer count was the second independent variable, which would give an indication of the effect of LIPUS on the viability of the odontoblasts in this *in vitro* model. Specific gene expression was studied after LIPUS application using real time polymerase chain reaction (RT-PCR). Dentin matrix protein-1 (DMP1), which is a major dentin non-collagenous protein that has a regulatory effect on development and differentiation of the odontoblasts, the initiation of hydroxyapatite nucleation and regulates the expression of osteocalcin, alkaline phosphatase, and dentin Sialophosphoprotein (DSPP). [20-25] Another non-collagenous protein that is

specific for the odontoblasts is the DSPP that cleaves into Dentine Sialoprotein (DSP) and Dentine Phospophyren (DPP), where the DPP interacts specifically with collagen and initiates hydroxyapatite crystals and controls the rate of crystal growth.[26-28] Investigating the effect of LIPUS on these two non-collagenous protein and collagen-I, which is the major structural component of the dentin matrix, also contributed in testing the first hypothesis. The second hypothesis was tested by evaluating the expression of transforming growth factor β1 (TGF-β1) that has a regulatory effect on the differentiation of odontoblasts[29,30] and upregulates odontoblast matrix secretion [31].

We evaluated the change in the RANKL/OPG ratio in the tooth slice organ culture (TSOC) model in response to the application of LIPUS. RANKL/OPG balance determines the osteoclastogenesis and odontoclastogenesis potential of the tissue, [32] and this balance or mechanism was found during early coordination of odontogenesis and osteogenesis during tooth development.[33] Furthermore this balance was found to be changing during the process of OIIRR favoring odontoclastogenesis and osteoclastogenesis.[34-36] By evaluating RANKL/OPG expression, we tested the third hypothesis, which will answer the question of whether LIPUS has an inhibitory or stimulatory effect on odontoclastogenesis mediated by these tumor necrosis factor-superfamily members (RANKL and OPG) inside the dentin-pulp complex.

In order to test the effect of LIPUS on OIIRR *in vivo* we conducted an animal study on beagle dogs. We used split mouth design to eliminate the individual variability nature of OIIRR. In this experiment, we tested the fourth

hypothesis in order to answer the question of whether LIPUS has a reparative/preventive effect on the OIIRR without side effects on the dental pulp and orthodontic tooth movement. That was tested by measuring the volume and number of resorption lacunae (RL), we used micro-computed tomography (μ-CT) images of the roots of orthodontically moved teeth. Moreover the roots were evaluated histologically to examine the effect of LIPUS on the pulp cellularity, cementum thickness and nature, PDL cellularity and the presence of hyalinization in the PDL. Immunohistochemistry was done to evaluate the pattern of distribution of odontoclasts on the root surface and osteoclasts on the alveolar bone around the orthodontically moved tooth. Clinical orthodontic tooth movement was measured to monitor any effects of LIPUS on tooth movement.

1.5 Organization of this Thesis:

This thesis was structured to address all the objectives and test the hypotheses in four chapters with an additional two chapters for the introduction and literature review and a final chapter for general discussion and final summary. Chapters three and four addressed the *in vitro* part of this study where we presented the short- and long-term effect of LIPUS on human tooth slice organ culture TSOC. We tested the effect of different periods of LIPUS application on the histological pattern of the dentin pulp complex. Also looked at the effect on the expression of collagenous and non-collagenous protein production by the pulp. Furthermore, we investigated the effect of LIPUS on the expression of

certain growth factors and the odontoclastogenesis controlling genes. These chapters (3 and 4) tested the first three hypotheses of this thesis.

The fifth and sixth chapters presented the radiographic and histological/ immunohistochemical outcome of the animal study, respectively. The animal study covered LIPUS effect on orthodontic tooth movement and root resorption variables from the periodontal ligament and dental pulp standpoints. Hard and soft tissue variables were measured and analyzed in this split-mouth study model. These two chapters (5 and 6) tested the fourth hypothesis of this thesis. Finally, the last chapter discussed the outcomes of all the performed experiments and the relationship between them; also we discussed the relevance of this research to clinical environment, the limitations of each experiment performed and final conclusion taken from our results.

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CHAPTER 2:

Background and Literature Review

2.1 Orthodontically induced inflammatory root resorption:

2.1.1 Definition:

Orthodontically induced inflammatory root resorption (OIIRR) is a form of the more general process called external root resorption. After enamel decalcification it is the second most common unavoidable side effect of orthodontic tooth movement. It is the consequence of a sterile inflammatory process that is extremely complex and multifactorial and happens in the periodontal ligament area when orthodontic force moves the root inside the PDL space and causes compression of the PDL tissue and ultimately occlusion of the blood vessels. It involves but not limited to the interaction between the following: the nature of applied force, tooth roots, bone, cells, surrounding matrix, and biologic messengers. Although, the outcome is frequently similar to other forms of root resorption, orthodontic root resorption is distinct from the other types of root resorption.

2.1.2 Occurrence and prevalence of tooth root resorption:

OIIRR is one of the most common side effects that accompany orthodontic treatment and comes second to the white spot lesion that results from enamel layer decalcification.[1-10] Also it was documented that it comes as the second most common type of root resorption after pulpal infection-related root resorption.[11] OIIRR severity usually classified based on histological or radiographic findings, histologically it's classified into: cemental or surface resorption with remodeling, dentinal resorption with repair (deep resorption) and Circumferential apical root resorption. Radiographically root resorption is graded numerically into the

following: 0 (No resorption) = no apical root resorption; 1 (Mild resorption) = slight blunting of the root apex; 2 (Moderate resorption) = moderate resorption of the root apex beyond blunting and up to one third of the root length; and 3 (Severe resorption) = excessive (severe) resorption of the root apex beyond one third of the root length. Most of the clinical studies used the radiographic grading in the classification of their findings, so in this part of the literature review we are using this classification.

Many studies concentrated on investigating the severity of root resorption and the affected teeth, maxillary incisors were found to be the most affected teeth by OIIRR in most of the prevalence studies.[12,13] Although resorption can occur without any orthodontic treatment, the association was very strong. The frequency of incisors showing any grade of root resorption increased from 15% before treatment to 73% after treatment, [14] and in another study from none before treatment to more than 80% after treatment.[15] This association includes the severity and extension too, where the number of teeth with moderate and severe root resorption increased from 1% before treatment to 25% after treatment.[14] In another prevalence study it was found that about 4% of orthodontic patients experience generalized resorption of the six anterior teeth of more than 3 mm.[16] Other studies reported that about 5% of adults[17] and only 2% of adolescents[18] are likely to have at least 1 tooth that resorbs more than 5 mm during treatment. The incidences of severe root resorption lacunae were more frequent in the apical one third.[19]

The process of OIIRR can occur quite early but the tissue has the ability to repair simultaneously or after. It has been shown that root resorption with loss of root length in humans can occur within 35 days.[20] With forces as low as 50 grams, areas of cellular cementum repair accompanied progressive apical resorption.[21] But this reparative process didn't reestablish the original root contours after orthodontic root resorption.[22] The ability of the body to repair root resorption lesions varied according to several factors like the continuity and magnitude of force.[23-30] Radiographic diagnosis of OIIRR is straightforward but the prediction is still challenging, fortunately some studies revealed some connection between early radiographic findings and the severity of OIIRR. Smale *et al* studied the occurrence of the OIIRR in the first 6 months of initiation of orthodontic treatment and they found that root resorption can begin in the early levelling stages of orthodontic treatment.[13] Orthodontic patients with detectable root resorption on periapical radiographs during the first six months of active treatment were found to be more susceptible to resorption in the following six-month period.[12] Minor OIIRR in the first 6 months of treatment were found to be indicative of progressive root resorption by the end of orthodontic treatment.[31]

Another way of predicting the severity of OIIRR was the presence of certain malocclusion characteristics of the patient and their anatomical borders. Significant correlation was found between the patient's overjet at the beginning of treatment and the amount of root resorption found in the maxillary central incisors, canines and lateral incisors.[32] Another study found correlation with changes in overbite but not with overjet.[33] In regard to anatomical factors, it has

been reported that severe root resorption occurs when the teeth roots are torqued against the palatal/lingual plates of bone. [34]

From this part of the literature we can conclude that OIIRR is a very common orthodontic side effect but fortunately sever progression of the process is not. Although the statistics are comforting for orthodontics practitioner; in case of progressive root resorption this can lead to compromised crown/root ratio with compromised function.[35] In severe resorption, where the crown-to-root ratio is adversely affected, mobility may occur and may require splinting in some patients.[36] This is one of the reasons that OIIRR is recommended to be included in every consent form when treating orthodontic patient, but this did not prevent the fact that OIIRR led to increased liability of orthodontists to malpractice claims.[37,38]

2.1.3 Predisposing Factors:

2.1.3.1 Genetic predisposition of root resorption:

Several prevalence and severity studies on OIIRR were conducted to investigate predisposing factors and highly susceptible teeth and individuals. It has been found that teeth during root formation are more resistant to OIIRR and follow-up shows that teeth with incompletely formed roots developed normally.[39] Despite the correlations found by different studies between OIIRR and orthodontic mechanics factors, Ong *et al* found that the difference between minor and severe external apical root resorption (EARR) was unlikely to be associated

with the kind of treatment received. But instead it was inseparably linked to the genetic makeup of the patient. [40]

Genotype was found to be a substantial influencing factor in the variability in severity of root resorption due to orthodontic force in rats.[41] Al-Qawasmi *et al* studied the genetic predisposition to external root resorption, and they reported that the TNFRSF11A locus, which encodes the receptor activator of nuclear factor-kappa B (RANK)- {which is one of the tumor necrosis factor TNF-receptor superfamily that plays a major role in osteoclastogenesis and odontoclastogenesis}, was associated with external root resorption.[42,43] Another study revealed that allele1 at the IL-1ß gene that is known to decrease the production of IL-1 cytokine *in vivo*, was found to significantly increase the risk of external apical root resorption.[44] This may support the hypothesis that increased root resorption associated with orthodontic tooth movement may be mediated by a decrease in the rate of catabolic bone modelling of alveolar bone which results in a prolonged stress and strain of the root against the alveolar bony socket.[42,45]

2.1.3.2 Orthodontic treatment biomechanics:

2.1.3.2.1 Type of tooth movement and root resorption:

During orthodontic treatment teeth are subjected to different kinds of tooth movement and usually a combination of them. The relationship between the type of orthodontic tooth movement and the occurrence of OIIRR emerged, thus multiple studies were conducted to explore this relationship. These studies gave valuable clues for understanding tooth-root resorption patterns after each type of

tooth movement. Intrusive and torque movements caught a lot of attention and were assumed to be the ones that results in more root resorption.

Orthodontic intrusion movement was linked to sever OIIRR due to the belief that all the orthodontic force is distributed over a small area of the PDL at the apex. So it was documented by several studies that intrusion with continuous forces induces root resorption. They noted severe changes towards the apical region of the root and in proportion to the magnitude of force applied.[46-48] The increased severity in the apical region can be attributed to the increased hydrostatic pressure in the apical PDL area.[49,50] On the other hand, orthodontic extrusion results in tension inside the PDL and not compression so it is assumed to produce less resorption. Han *et al* (2005) found more root resorption results from intrusive forces compared to extrusive ones, yet there was root resorption caused by extruding teeth.[51] Extrusive orthodontic force behaved like intrusive orthodontic movement where the severity of root resorption caused by it was affected by the force magnitude.[52] Only one animal study found no association between the severity of resorption and the intrusive force magnitude.[53]

Orthodontic torque movement of the teeth for a longer time period and with a higher magnitude of applied moments showed a higher degree of root resorption in lacunae width as well as in depth.[54,55] Others conducted studies to test the effect of rotational tooth movement on root resorption. The results showed that the resorption severity was correlated with the treatment duration[56] and applied force magnitude.[57] From the reviewed studies about the kind of tooth movement, as a factor it will just determine the areas of tension, compression and shear inside the

PDL which is responded to differently and ultimately will only affect the distribution of root resorption and unlikely will influence the severity. Also, we can get that force magnitude and the duration have more weight in the process of root resorption. In the next two sections we will review these two factors and their relationship to OIIRR.

2.1.3.2.2 Force magnitude, regimen and root resorption:

In clinical orthodontics, clinicians aim for an optimum force that will move teeth into the planned position and produce minimum or no harm. The force magnitude is one of the factors that were investigated heavily in order to reach ideal outcome by conducting painless treatment with less root resorption. In the search for the ideal clinical orthodontic force, Reitan proposed that the ideal spring force should be constant over time and independent of the amount of tooth movement that occurred.[58] That was in regard to clinical efficiency but from experiments on animals [59-61] and humans[62] continuous forces led to more severe root resorption than non-continuous forces. Also Acar *et al* found from electronmicrographs of the roots of 22 first premolars which were experimentally moved for 9 weeks that less root resorption occurred on the discontinuous force side.[63] Moreover, Weiland (2003) reported that the amount of root resorption was significantly greater with higher and continuous force group of orthodontically moved human premolars.[64] Another form is intermittent force which was found to induce high RANKL expression in periodontal ligament cells[65] which is associated with the increased potential of root resorption.[66-68] But clinical studies

did not prove the hypothesis of increased OIIRR due to intermittent force as in the case of continuous force.

A number of studies on animals [69] and humans [70,71] concluded that force magnitude as a single factor is probably not decisive for root resorption, but significant number of studies provided evidence of association between the severity of root resorption and the increase in force magnitude.[47,57,61,71-74] In a review by Weiland, they looked at the correlation between OIIRR and forces and the conclusion was that the combination of force magnitude and duration of application appears to be a key factor in root resorption. Forces that are heavy enough to cause necrosis of the periodontal ligament and last long enough to prevent the root from repairing the damaged surface seem to be dangerous and should be avoided. [75]

2.1.3.2.3 Treatment duration and root resorption:

A factor that interests orthodontists clinically is the correlation between OIIRR and the overall orthodontic treatment duration, so many studies investigated this relationship and a strong correlation was found.[61,76-79] In a study on rats, this strong correlation between the incidence and severity of root resorption and the duration of tooth movement was only found in the adult rats.[80] Kurol *et al* found in a 7-week tooth movement study apical root resorption was evident in all test teeth by the third week but after the seventh week, the test teeth showed on average of about 20 times the resorption in the control teeth.[81] Unfortunately, OIIRR does not stop when the patient goes into retention, which is

the period of time after orthodontic treatment being finished, and the teeth are held in place passively. It was found that teeth are still prone to additional root resorption during retention and relapse but it was also linked to the original treatment duration.[82]

In summary, orthodontic mechanics play a role in the incidence and severity of OIIRR but no single variable can be isolated as the major risk factor. However, force magnitude and duration are key factors that were revealed to have strong influence on OIIRR by previous studies. The combination of a force with certain magnitude for an adequate period of time will highly influence the process of OIIRR. This force has to be strong enough to cause necrosis inside the PDL that will initiate the process of root resorption; in addition it has to be maintained for long period of time as in the case of continuous force and longer treatment duration which will minimize the chance of repair by the body.

2.1.4 Physiology of root resorption and prophylactic strategies

2.1.4.1 ***Physiology of root resorption***

Root resorption, although much studied, is a poorly understood phenomenon in which the periodontium is the principle active biologic unit where the cascade of events occur after applying orthodontic force.[83] So after applying a force that is strong enough to occlude blood vessels in the PDL, hyalinization of the PDL happens and ultimately bone and root resorption occurs. Fortunately bone resorbs more readily than the root by this process.[84] So an association between OIIRR and local damage of the PDL due to over-compression was found.

Retardation and stagnation of the blood flow in compression zones lead to sterile necrosis within the PDL around which the bone and root resorption starts.[85-88] This can be explained by the presence of more root resorption in the area of compressive forces within the PDL than combined compressive and tensile forces.[89] Subsequently due to the chemotactic factors resulting from the formation of necrotic tissue, phagocytic cells such as macrophages, foreign body giant cells and osteoclasts respond to these signals by removing the damaged tissue.[90-92]

From light and electron microscopic studies on the initial process of root resorption and removal of the hyalinized tissue it has been found that during the remodelling process, root resorption may occur as an adverse effect of the cellular activity associated with removal of the necrotic tissue. [93,94] The initial access of resorptive cells to the root surface occurs at the periphery of the necrotic zone. The cells initially are mononucleated, stained negatively to tartrate resistant acid phosphatase (TRAP) indicative of non-osteoclastic lineage, and resemble macrophages or fibroblasts.[95] Also Kurol and Owman-Moll found hyalinized zones were recorded opposite to intact root surface (54%) or just apical or coronal to an area of resorption (45%). [96] Root resorption beneath the main necrotic zone takes place at a later stage, during which multinucleated TRAP-positive cells remove the bulk of necrotic PDL tissue then resorbs the outer layer of adjacent root cementum. These cells has no ruffled borders but multinucleated TRAP-positive cells with ruffled borders can be found in the deeper root resorption lacunae.[95]

During the process of bone resorption the active osteoclasts show a high content of TRAP. [97,98] Many histochemical studies demonstrated that a specific isoenzyme in the cytoplasm of the multinucleated OC could be distinguished from that in other bone cells by its resistance to being inhibited by tartaric acid. Moreover, osteoclast-like cells and their precursors were found to stain positively for TRAP. [99-103] TRAP stain was used for identification of cells involved in orthodontic tooth movement (osteoclasts) and root resorption (odontoclasts, cementoclasts). [98,104,105] Brudvik and Rygh found that TRAP enzyme is of significant importance for the removal of necrotic periodontal ligament tissue and of the superficial part of root cementum.[95] During that process dentinal tubules could be exposed, a phenomenon being considered as a stimulus for attracting progenitor cells for differentiation and fusion into clast cells, and this was supported by the presence of higher concentrations of anti-HDE (human dentin extract) antibodies in the more severe root resorption cases[106].

Brudvik and Rygh showed in some of their studies that the thinning of the cementum layer leaves a naked unprotected dentin where the cells resorbing it had a ruffled border surrounded by clear zones and these cells were odontoclasts.[95,107,108] They hypothesized that the multinucleated cells that resorbed the root surface beneath the hyalinized tissue in the PDL were derived from monocytes and macrophages that invaded this glass-like necrotic tissue.[95] Udagawa *et al* [109] study was supportive when they found that clast cells that caused resorption lacunae on dentin slices in a cell culture model were derived from mature monocytes and macrophages when sufficient environment provided.

Moreover, the role of dentin exposure in root resorption process was supported by the study of Sasaki *et al* in which they clearly indicated that odontoclasts develop ruffled border structures only when they are in direct contact with mineralized dentin surfaces both *in vivo* and *in vitro.*[110]

The pulp-dentin complex is thought to be affected by orthodontic tooth movement since some researchers have demonstrated that orthodontic treatment alters pulpal blood flow [111-114] and the activity of the odontoblastic layer, [112,115] generating an obliteration of the pulp space by producing tertiary dentin.[116] Although orthodontic tooth movement has several effects on the dentin-pulp complex, research showed that there is no correlation between endodontically treated teeth and root resorption due to orthodontics. [117-119] Furthermore, TRAP-positive enzymatic reaction was also found to penetrate the dentinal tubules.[120]

At the molecular level the mechanism of root resorption process is still unknown but some studies introduced hypotheses in regard to the signaling by some chemical mediators that may participate in the bone remodelling process. It was found that application of orthodontic force induces the synthesis of proinflammatory cytokines IL-1β and IL-6, which plays important role in bone resorption during the application of orthodontic force.[121] In another animal study, it has been shown that there was an increased expression of IL-1α and TNF-α as early as one day after mechanical force application at both compression and tension areas. But the cytokine expression in the compression area was always more intense than in the tension area.[122] A study on IL-1β knockout mouse supported the hypothesis that excessive root resorption associated with

orthodontic tooth movement may be mediated through a decreased rate of alveolar bone resorption resulting in prolonged stress and strain of the tooth root against the alveolar bone.[44,45]

The hypothesis that OIIRR may be mediated through impairment of alveolar resorption, resulting in prolonged stress and strain of the adjacent tooth root due to dynamic functional loads, [42,45,123] contradicts with the hypothesis that increased severity of root resorption due to orthodontic treatment is related to increased alveolar bone resorption.[124] But root resorption can be still related to the reduced rates of bone resorption at the PDL interface of the root and the alveolar socket, because that can result into a prolonged resorption/inflammation inductive phase associated with compressed necrotic areas in the PDL prior to the occurrence of alveolar bone resorption.[43]

Tumor necrosis factor superfamily received a lot of interest while investigating the mechanism of OIIRR due to two reasons, the first is the hereditary predisposition of OIIRR by genes encoding for some members of this family and the second is its known role in the process of bone resorption. In bone biology the osteoclastogenesis and bone resorption are regulated by production of the tumour necrosis factor TNF receptor-ligand family members which includes, osteoprotegrin (OPG) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) by osteoblast/stromal cells.[125] RANKL is considered as a member-associated cytokine and a bone microenvironment-associated soluble factor that when bound to receptor activator of NF-κB (RANK) on osteoclast precursor cells it promotes osteoclastogenesis.[126-128] Osteoclastogenesis was found

to be dependent on the balance between RANKL and OPG expression in osteoblasts, where OPG decreases osteoclastogenesis and RANKL increases it.[129,130]

OPG/RANK/RANKL pathway controlling the osteoclastogenesis and odontoclastogenesis was found to exist in physiologic root resorption in deciduous teeth. [131] In another study on developing rats, these TNF superfamily receptors and ligands were detected in the developing rat molar. Lossdorfer *et al* immunohistochemically detected granular cytoplasmic RANKL in odontoblasts, pulp fibroblasts, PDL fibroblasts, and single odontoclasts in human resorbing deciduous teeth, which indicates that odontoclasts had an autocrine-paracrine role.[132] Regarding the role of these mediators in the root resorption prospective, it has been found that cementoblasts also express RANKL and OPG and can cause cementoclast formation by increasing the RANKL/OPG ratio with the parathyroid hormone PTHrP.[133] RANKL and OPG levels were seen to increase in the environment during the application of heavy forces and severe root resorption.[66-68,134] Moreover, compressed PDL cells obtained from patients with severe external apical root resorption produced OPG and RANKL differentially where it produced large amounts of RANKL and low of OPG, which stimulated osteoclast formation.[68]

From another prospective, the OIIRR was linked to immune response and its mediating factors. Alhashimi *et al* reported that fibroblast-; macrophage- and dendritic-like cells express CD40, which is a cell surface receptor that belongs to the tumor necrosis receptor family (TNF-R) in normal rat PDL. Cellular responses

mediated by CD40 are triggered by its counter receptor, the CD40L, which is defined as a type II membrane protein that is also a member of the TNF gene family. This study illustrated that CD40 expression in these cells was markedly expressed after application of orthodontic force. Moreover, CD40-CD40L interactions appear to play a role in the immune responses mediated by periodontal cells during orthodontic tooth movement which may induces T-cell activation and generates the harmful inflammatory reactions that results in bone resorption or an immune response with anti-inflammatory mediators that may play a defensive role against root resorption.[135]

In regard to the structural components of cementum and its effect on the process of OIIRR, Osteopontin (OPN) deficiency in mice was investigated. Osteopontin is a bone matrix protein involved in the regulation of cell function and one of the major non-collagenous components of the cementum layer. Osteopontin deficiency significantly decreased the force-induced increase in the odontoclasts population and reduced root resorption. Force application induced an increase in the number of TRAP-positive cells in the alveolar bone on the pressure side which was defined as osteoclasts, while the levels of the osteoclastic cell numbers in such alveolar bone were similar between the Osteopontin-deficient and Wild-Type mice, which indicates that Osteopontin deficiency suppresses tooth root resorption due to force application. [136]

2.1.4.2 ***Prophylactic strategies***

Cementum is described as a nonuniform mineralized connective tissue which is found in several distinctly different types on human dental roots.[137] Two types of cementum can be classified according to the presence or absence of cells as cellular and acellular cementum.[138] Cellular cementum is less mineralized and is deposited around the apical third of cementum, whereas acellular cementum covers the coronal two thirds of the root. Acellular cementum consists of only mineralized layers.[138,139] Cervical and middle third cementum have greater hardness and elastic modulus than that of the apical third [140,141]; apparently because of the variability in mineral content between cellular and acellular cementum. It was also found that hardness was positively correlated to the amount of mineralization.[142,143] Therefore, it will be more likely that the harder the cementum will have less resorption.[21] Moreover, the density and hardness of cementum and dentin may retard the root resorption process.[144]

Studies were performed to investigate the changes in physical properties cementum after the application of controlled orthodontic forces.[145,146] It was found that the application of orthodontic forces causes alteration in the mineral content of cementum, and resulted in a trend of increased the mineral content (Ca, P, and F) of cementum at various areas of the PDL compression. Also, there was an overall decrease in the Calcium concentration of cementum with the application of heavy orthodontic forces that corresponds to areas of PDL tension.[147]

Despite that cementum microstructure and mineral content has a role in its resistance toward OIIRR, cementum formative cell layer is considered to be the

first mechanical line of defense against this process. This layer is thought to be the only barrier that protects it from a resorptive attack by osteoclastic cells.[148-150] It has been documented that the uncalcified mineral tissues, osteoid, pre-cementum and pre-dentin are more resistant to resorption process and may initially prevent the loss of tooth hard tissue.[21] However, continuous pressure will lead to a breach of the naturally protective formative cell layer which will allow the exposure of the mineralized tissue to the periodontal matrix cells and their inflammatory byproducts.[21,150] This is possible also when the cementum is mechanically damaged.[148]

2.1.5 Root resorption treatment attempts:

The best start of treatment is prediction and early diagnosis, because prediction of OIIRR is not accurate; this will leave clinicians with the option of early detection. A precautionary clinical recommendation was proposed for maxillary incisors with an enhanced risk of root resorption where clinicians should do a 3-month radiographic follow up.[151] Several treatment attempts for OIIRR have been done in the last three decades. The management techniques varied from treatment regimens to treatment mechanics modification that allows the root to repair itself.

In regard to allowing self repair, Harry and Sims (1982) found after severe resorption due to intrusion that healing occurred after 70 days along with the continuing root resorption in cases with continuous orthodontic force.[152] Vardimon *et al* (1993) in a study on monkeys reported that retention after palatal

expansion helped in root resorption repair with nonfunctional and functional cementum formation.[153] In a human study Owman-Moll, 1995 showed evidence of root functional repair, with the total surface of the resorption cavity walls covered with varying thicknesses of cementum after retention (33% to 40%). However, individual variations in healing response were large.[28]

Chemical regimens varied from bone remodeling inhibitors to some proteins applied in an attempt to control or prevent root resorption. Bisphosphanate was applied subperiosteally adjacent to rats' teeth roots in a study by Igarashi *et al* 1996. They reported that the topical administration of risedronate caused a significant and dose-dependent inhibition of root resorption after orthodontic force application in rats.[154] Keum *et al*, (2003) used topical corticosteroid on the root surface of avulsed rat teeth. They found topical use of dexamethasone might reduce the degree or rate of progressive root resorption secondary to traumatic avulsion. Unfortunately a higher chance of teeth ankylosis was noticed in the dexamethasone treated group.[155] Sakallioglu, *et al* (2004) showed non-significant results for acellular cementum formation on the root surface of dogs' teeth after the application of enamel matrix protein. Although the resulting cementum was acellular, the lack of statistical significance may be due to the small sample size (4 dogs). [156] Aqrabawi and Jamani (2005) used a more invasive approach by filling root canals with calcium hydroxide. This approach succeeded in arresting the resorptive process; however no new dental tissues were formed. [157]

Due to the invasive and challenging methods of local application of proposed chemical agents and the risks and side effects accompanying their systemic administration, they did not reach the point of clinical acceptability especially in orthodontics. After the finding of dental tissue formation in rabbits by Low Intensity Pulsed Ultrasound (LIPUS) [158], El-Bialy *et al* conducted an *in vivo* study where they demonstrated that ultrasound stimulation which consisted of a 200-microsecond burst of 1.5 MHz sine waves repeating at 1 kHz that delivered 30 MW/cm^2 incident intensity enhances the healing process of OIIRR due to tipping movement in humans. A firm attachment of acellular cementum to the root dentin with functional organization of its collagen fibers was noted.[158] Similar results were found in an animal model, and the effect was linked to an increase in the OPG/RANKL ratio.[159] This effect could be due to the fact that LIPUS treatment induced more cementum and dentin matrix production by cementoblasts and odontoblasts respectively.[158]

2.2 Low Intensity Pulsed Ultrasound LIPUS:

2.2.1 Definition and uses in the medical and dental field.

Ultrasound is an acoustic pressure wave at frequencies above the limit of human hearing, which is transmitted into and through biological tissues and is being used widely in medicine as a therapeutic, diagnostic, and operative tool.[160,161] Therapeutic ultrasound and some operative ultrasound use intensities as high as 1 to 3 W/cm^2 and can result in considerable heating of the living tissues. Therapeutic ultrasound is widely used, mainly in sports medicine and myofunctional therapy, in the reduction of joint stiffness, muscle pain and spasms,

and to improve muscle mobility.[162] Also therapeutic ultrasound can become a powerful non-viral method for the delivery of genes into cells and tissues.[163,164]

LIPUS has been reported to be effective in liberating preformed fibroblast growth factors from a macrophage-like cell line (U937) and stimulates angiogenesis during wound healing, [165] enhances bone growth into titanium porous-coated implants, [166] enhances bone healing after fractures[167,168], enhances bone formation after mandibular osteodistraction[169-172] and enhances healing of osteoradionecrosis.[173-175] Other studies showed that therapeutic ultrasound stimulates the expression of bone proteins like osteonectin, OPN, bone sialoprotein, and this stimulatory effect is dose dependent.[176,177] Also, LIPUS can enhance mandibular growth in growing patients with Hemifacial Microsomia.[178] It has been reported that LIPUS enhances the formation of lower incisor apices and accelerates the rate of eruption of teeth in rabbits.[179] Moreover, ultrasound was found to have an anti-inflammatory action.[180-182]

2.2.2 LIPUS effect on root resorption:

El-Bialy *et al* studied the effect of LIPUS on the healing of orthodontically induced root resorption in 12 patients. They reported that LIPUS minimized root resorption and accelerated healing of the resorption by reparative cementum over 28 days of simultaneous tooth movement and daily LIPUS application.[158] In a recent study on rats, LIPUS was found to regulate the osteoclast differentiation through the OPG/RANKL ratio and initiated reparative effect on orthodontically induced root resorption.[159] Dalla-Bona *et al* (2006) demonstrated that LIPUS

affects cementoblasts by regulation of some genes-related protein in vitro. They demonstrated that an intensity of 150 mW/cm^2 was most effective for upregulation of calcium content and transcript of alkaline phosphatase (ALP) by cementoblasts, which plays an important role in mineralization process, but LIPUS had no effect on cell proliferation.[183]

In another study by Dalla-Bona *et al,* they found that high-intensity ultrasound increased the OPG synthesis by cementoblasts, while RANKL levels were unaffected. RANKL synthesis was quite low in both groups. These changes in the OPG/RANKL ratio can be anticipated to result in an active production of inhibitory factors which decreases the formation and activity of cementoclasts, and subsequently a decrease in root resorption. Moreover they found both low- and high intensity ultrasound applications enhance cementoblast transcripts for ALP and COL-I *in vitro*. Furthermore, OPG at the protein level, collagen synthesis and ALP activity were found to be significantly enhanced only by high-intensity ultrasound. The stimulation of collagen synthesis indicates a direct role in cementum matrix formation and the upregulation of OPG synthesis indicates an inhibitory effect on cementoblast-mediated osteoclastogenesis. However, they noted that US with 150 mW/cm^2 may be harmful to the dental pulp cells which may need more *in vivo* investigation.[184]

In a short term *in vitro* study on odontoblast-like cells, low frequency ultrasound (30kHz) resulted in an increase in the expression of collagen type I, OPN, transforming growth factor-β1 (TGF β1) and the heat shock protein (hsp) 70. The expression of the small heat shock proteins (hsp) 25/27 showed a two to

six-fold increase following ultrasound group without significant effects observed in the expression of ALP and core-binding factor A1 (CBFA1/Runx2) (CBFA1 is an essential transcription factor for osteoblastic differentiation and osteogenesis) expression, where both when highly expressed are indicative of more differentiation and less proliferation.[185]

2.2.3 Hypothetical mechanism of action

Despite multiple studies in the field of biological effects of the therapeutic ultrasound, the physical process through which low level ultrasound interacts with living tissue remains unknown. The difficulty in resolving this issue lies in the complex response of living tissue to these high frequency acoustic stimuli. While passing through the tissue, its energy is reduced due to absorption by tissue layers at a rate proportional to the density of the tissue. This differential absorption may play a critical role in targeting the ultrasound to the cells present inside and around the hard tissue (e.g. bone and teeth).

Wide ranging *in vitro* and *in vivo* studies have been conducted to investigate the biologic mechanism(s) responsible for the observed ultrasound augmentation of osteogenesis and fracture healing. One of the first such *in vitro* studies reported that ultrasound induced changes in rates of influx and efflux of potassium ions in rat thymocytes.[186] Ryaby *et al* reported that low intensity ultrasound increased calcium incorporation in differentiating cartilage and bone cell cultures.[187,188] Also calcium uptake was increased in fibroblasts with reversible efflux of the calcium after exposure.[189] Another study has concluded that ultrasound-stimulated

synthesis of cell matrix proteoglycan in accelerated fracture healing is mediated by intracellular calcium signalling.[190] *In vitro*, US at a spatially averaged temporally averaged intensity I_{SATA} of 20–30 mW/cm^2 has been shown to modulate adenylate cyclase activity,[187,188,191-193] transforming growth factor beta (TGF-b) synthesis,[192,193] bone morphogenic protein (BMP) effects,[191] and parathyroid hormone (PTH) responses.[193]

Tsai *et al* concluded in their study of ultrasound treatment of injured tendon that ultrasound stimulates the expression of collagen (type I and type III) is likely to be mediated by the upregulation of TGF-β.[194] It was demonstrated in another study that pulsed ultrasound exposure could increase TGF-β secretion by osteoblasts, and decreased the concentration of IL-6 and TNF-α in the culture medium which would prevent bone loss.[195] Another study demonstrated an increase in the total ALP amount in the culture medium, and tumour necrosis factor-α in ultrasound-stimulated bone cells which would be stimulatory for the process of bone healing.[196]

Zhou *et al* found in the primary fibroblasts in cultured human skin fibroblasts that US-induced proliferation involves the activation of β1 integrins and RhoA/ROCK and Src-ERK intracellular signaling cascade. However, integrin activation and cell proliferation resulted from the acoustic pulsed energy did not involve activation of the EGFR, which demonstrates that this mechanical stimulation triggers a specific signaling platform.[197]

Hayton *et al* hypothesized that US causes adenosine 5'-triphosphate (ATP) release by osteoblasts *in vitro* which may accelerate fracture healing by enhancing

osteoblast proliferation and increasing the expression of RANKL and decreasing OPG expression by osteoblasts to promote osteoclastogenesis.[198] Furthermore, Mukai *et al* reported that LIPUS promotes the proliferation and differentiation of chondrocytes and suggested that LIPUS effect in chondrocytes was mediated by TGF-β1.[199] Doan *et al* [200] and Reher *et al* [201] showed US at ISATA (20 mW/cm^2) stimulates the macrophage release of vascular endothelial growth factor (VEGF), a growth factor associated with endothelial cell proliferation and migration. Also, US with an intensity of 50 mW/cm^2 significantly increased chondrocyte expression of genes coding for aggrecan *In vitro*.[202,203] These effects are suspected to be the result of enhanced uptake of calcium with US exposure.[204]

Tsai *et al* reported that optimum ultrasound duration and intensity for bone fracture repair were obtained by applying ultrasound with 0.5 W/cm^2, 1.5 MHz for 15 min/day.[205] Based on these results, most of the recent commercially available LIPUS devices were manufactured. Of these devices, El-Bialy *et al* used the Exogen device (Smith & Nephew, Memphis, TN, USA) for stimulation of tooth eruption and formation in rabbits as well as in repairing OIIRR in humans. Tsai *et al* [205], also Tanzer *et al* [166] reported that LIPUS effect is optimum during the first 2-3 weeks of treatment.

2.3 Tooth slice organ culture:

Attempts to culture odontoblasts *in vitro* as a cell line have shown that contact between them and the dentin matrix as in the normal histological architecture inside the dental pulp is required to maintain their normal

morphology and secretory activity.[206] A model allowing long-term culture of the dentin-pulp complex would be valuable for the study of molecular and cellular processes involved in the dentin-pulp complex during the application of LIPUS. A human tooth organ culture model of the dentin-pulp complex has been reported in which cell viability was maintained for longer periods. This model had some limitations toward modifying the growth conditions.[207]

Another approach overcame some of those problems was used for the culture of embryonic dental papilla, where they embedded the cells in a semi-solid agar based medium and grown at the liquid-gas interface on filters in Trowel Type cultures. That technique may was adapted to a more successful and longer-term culture of the dentin-pulp complex from mature rodent teeth to study the effect of TGF-ß1 and BMP2 on the differentiation of odontoblasts.[208]

2.3.1 Technique:

Sloan *et al* developed tooth organ culture technique in rats. The technique is described briefly as follows [209]. Upper and lower incisor teeth were dissected from 28-day-old male Wistar rats euthanized by cervical dislocation. The teeth were then placed in sterile washing medium consisting of DMEM containing antibiotic/antimycotic (1000 units/mL penicillin G sodium, 10µg/mL streptomycin sulphate and 25µg/mL amphotericin B), and subsequently embedded in dental impression compound through which transverse sections (2 mm thick) were cut with a segmented, diamond-edged rotary saw cooled with washing

medium. The tooth slices were immediately placed and washed in a sterile washing medium.

The sections of incisors were washed several times in the washing medium at 37°C after cutting, then transferred to individual wells of a 96-well dish. Culture medium (100 ml) containing DMEM, vitamin C (0.15 mg/ml), 10% heat inactivated fetal calf serum, L-glutamine (200 mM), 1% penicillin/streptomycin solution and 1% low melting-point agar maintained at 37°C was added to each well while warm then allowed to cool and harden. After reaching a semisolid state, the embedded tooth slices were transferred to a sterile Millipore (mixed esters of cellulose acetate and nitrate) filter floated on the surface of 4 ml DMEM in 35 × 10 mm Petri dishes. The tooth slices were cultured at 37°C in an atmosphere of 5% CO_2 in air, in a humidified incubator for up to 14-21 days, medium was changed every 2 days.[209]

2.3.2 Role in studying the pulp-dentin complex

The importance and usefulness of this model in studying the pulp-dentin complex was demonstrated by several studies on the pulp and dentin response to different restorative materials or chemical materials and even orthodontic forces. In 1998 Sloan *et al* studied the process of dentinogenesis in rat incisors for up to 14 days *in vitro*. They demonstrated that the dentin-pulp complex from mature rodents can be cultured successfully for substantial periods of time and can be useful in studying the process of dentinogenesis and dental tissue repair.[209] In another study Sloan and Smith (1999) tested the effect of transforming growth

factor-β (TGF-β) isoforms on dentinogenesis and extracellular matrix secretion by the odontoblasts; and they found that TGF-β1 and - β3 can stimulate secretion of extracellular matrix by odontoblasts, and that TGF- β3 may have inductive effects on pulpal cells.[210]

Murray *et al* (2000) tested the effect of different materials included in the formulation of dental products, and they ranked them based on the amount of pulpal injury.[211] Moseley *et al* (2003) studied the effect of fluoride on dentin matrix formation and composition. They demonstrated incorporation of fluoride into the dentin matrix during culture caused an increase in the Ca/P ratios of the mineral and a more diffuse pattern of tetracycline incorporation which implied that alterations in dentin formation during fluorosis are a consequence of disruption to the mineralization process.[212]

Saw *et al* (2005) conducted a study comparing cell line (fibroblasts) and tooth slice cultures (28-day old male Wistar rats) response to composites polymerized with two types of light cures. Discrepancy in result presentation in this study highlights the risk involved when relying on a single test method for cytotoxicity assessment, and recommended the use of different culture models and then proceed to a more clinically relevant biological system that stimulates the *in vivo* environment for confirmation.[213]

As a variation of the original tooth slice organ model Dhopatkar *et al* (2005) introduced a novel in vitro culture model to investigate the reaction of the dentin–pulp complex to orthodontic forces. The forces induced a cellular response in the pulp tissue characterized by alteration in gene expression and proliferation of

fibroblasts.[214] This model was recently used in studying the effect of LIPUS on the dentin-pulp complex during the application of orthodontic force on it. It was found that LIPUS can influence remodelling of the dentine-pulp complex and periodontal tissue during orthodontic force application.[215]

In the previous studies the slices were obtained from animal teeth (28-day male Wister rat) but in the following studies the slices were obtained from human teeth. Dobie *et al* (2002) tested the effect of alginate hydrogels and TGF- β1 on slices obtained from human third molars. TGF-β1 can cause both induction of odontoblast-like cell differentiation and upregulation of extracellular matrix secretion by them in the dentin–pulp complex. Alginate hydrogels was suggested to be an appropriate matrix for dental tissue regeneration and may be useful for delivery of growth factors like TGF-βs, to enhance the regenerative capacity of the dental pulp.[216] Graham *et al* (2006) investigated the mechanism behind the effect of Calcium hydroxide (Ca $(OH)_2$) and EDTA. They hypothesized that calcium hydroxide ($Ca(OH)_2$) may apply its effects on dentin regeneration by mechanisms involving cell signalling resulting from dissolution of bio-active molecules from the dentin matrix.[217]

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CHAPTER 3:

Long-term effect of Low Intensity Pulsed Ultrasound on a human tooth slice organ culture

3.1 Introduction:

The dentin pulp complex is a key player in the formation of the tooth structure during development and has a protective nature when assault is introduced to the tooth by forming tertiary dentin (reparative dentin). Like other tissues of interest attempts to culture odontoblasts *in vitro* have been done, but it has been shown that their contact with the extracellular dentin matrix is crucial for maintaining their phenotypic morphology and secretory activity.[1] A human tooth organ culture model for the dentin-pulp complex has been reported in which cell viability was maintained for different periods. In this model, slices were cultured in the base of a dish, which has limited experimental manipulation of growth conditions.[2] This model was applicable in testing the cytotoxicity of dental restorative materials and the stimulatory effect of growth factors on the dentin-pulp complex.

Sloan *et al* reported that the dentin-pulp complex from mature rodent tissues could be cultured successfully for considerable periods of time and can grant a useful model for studying dentinogenesis and tissue repair in the dentin-pulp complex.[3] Follow-up studies tested the effect of transforming growth factor-β (TGF-β) isoforms on dentinogenesis and extracellular matrix secretion by odontoblasts and found that TGF-β1 and - β3 can stimulate secretion of extracellular matrix by odontoblasts and that TGF- β3 may have inductive effects on pulpal cells.[4] Saw *et al* conducted a study comparing cell line (fibroblasts) and tooth slice cultures (28-day old male Wistar rats) response to composites polymerized with two types of light cure. The discrepancy in results they

presented brought out the risk of relying on the cell line culture method alone for cytotoxicity assessment.[5]

Low Intensity Pulsed Ultrasound (LIPUS) has been widely used clinically and for biological studies. It uses intensities low enough to be considered neither thermal nor destructive. Typically, an intensity of 30 mW/cm^2 is used for LIPUS. The mechanical wave generated is transmitted through and into living tissue as acoustic pressure waves where this energy is absorbed at a rate proportional to the density of the tissues in which it passes through. The micromechanical strains produced by these pressure waves in biological tissues are assumed to initiate biochemical events that affect hard and soft tissue activity. This form of therapeutic ultrasound has already been very well established for the enhancement of bone fracture.[6] Due to the non-invasive stimulatory effect of LIPUS on callus formation and maturation, research has extended into studying the effect of LIPUS on dental and periodontal tissues. At an *in vitro* level, Dalla-Bona *et al* demonstrated that low- and high-intensity ultrasound affects cementoblasts by regulation of mRNA expression of alkaline phosphatase (ALP), which plays an important role in the mineralization process, but ultrasound had no effect on cell proliferation.[7] In cultured human periodontal ligament cells, LIPUS enhanced ALP activity, collagen synthesis, and Runx2 expression, which provide important insight into the promotion of early cementoblastic differentiation of immature cementoblasts.[8]

In this experiment we investigated the effect of LIPUS on the dentin-pulp complex *in vitro* model to demonstrate any changes at the cellular or molecular

levels in the dentin-pulp complex. This *in vitro* model will maintain the close relationship between the odontoblastic cell layer and the dentin matrix; however, at the same time it will give us the flexibility of investigation and control of the study design in an *in vitro* experiment while exploring the effect of LIPUS on the dentin pulp complex.

3.2 Materials and Methods:

3.2.1 Samples and sample collection:

Ethical approval was obtained from the Health Research Ethics Board at the University of Alberta. Samples were obtained from 23 adolescent orthodontic patients requiring extraction and with a mean age of 14y-5m ± (2y-3m). Inclusion criteria for the selected teeth were that the tooth should be sound and have an open apex on the panoramic x-ray film. Ninety-two premolars were obtained and 8-11 slices were obtained per tooth. The samples were divided into 10 groups where LIPUS was applied daily for 5 days in four groups of application duration (5, 10, 15 and 20 minutes) with one control group. The other five groups were prepared following the same methods but with LIPUS application only once at the beginning of the culture.

3.2.2 Tooth slice organ culture:

After extraction, teeth were placed in a sterile washing medium consisting of DMEM containing antibiotic/antimycotic (1000 units/mL penicillin G sodium, 10µg/mL streptomycin sulphate and 25µg/ml amphotericin B). The teeth were sectioned transversely into 600 µm-thick sections with a 0.006" diamond wafer saw (ISOMET® Wafering Blade, Series 15HC, 3" x 0.006" x 1/2") (Buehler, Whiteby, Ontario, Canada) and cooled with sterile PBS. Tooth slices were immediately placed in sterile washing medium and washed several times at 37°C and then transferred to individual wells in a plastic 6-well plate containing 4 ml of culture media. The culture medium contained DMEM, vitamin C (0.15 mg/ml), 10% heat inactivated fetal calf serum, L-glutamine (200 mM), and 1% penicillin/streptomycin solution. Tooth slices were cultured at 37°C in an atmosphere of 5% CO_2 in air, in a humidified incubator, and the medium was changed after 24 hours and every 2 days afterwards.

3.2.3 Ultrasound application and calibration:

Custom-built ultrasound device that provide adjustable output parameters and long-term operation stability was used (Smilesonica Inc., Edmonton, AB, Canada). The LIPUS devices were set to generate ultrasound pulses with a repetition rate of 1 KHz. Each pulse has a square envelop with duration of 200 microseconds and a pulse frequency of 1.5 MHz. For each device, the ultrasound transducer has an emitting surface area of 3.9 cm^2 and it generates a temporal average ultrasound power of 120mW (or a temporal average ultrasound intensity

of 30 mW/cm^2). The transducer was applied sequentially below each of the 6-well plates using high viscosity gel (National Therapy Products Inc., Woodbridge, ON, Canada) in between as a coupling media inside the incubator. The amount of signal attenuation after passing through the plate material was measured in the lab and it was found to be 4% of the total output power. At the beginning and at the end of the experiments the ultrasound devices were inspected for consistency of electrical waveforms (1 KHz modulation, 200 microseconds pulse duration, and 1.5MHz pulse frequency) using TDS1012C-EDU digital oscilloscope (Tektronix, Canada), and calibrated for ultrasound intensity of 30mW/cm^2 using an ultrasound power-meter (model UPM-DT-1AV from Ohmic Instruments, Easton, MD, USA). The calibrations at the beginning and at the end of the experiments confirmed that the ultrasound devices provided stable ultrasound power output and maintained the desired parameters during the experiment. Culture media were monitored for thermal changes due to the application of LIPUS using TL1A Series High Accuracy Digital Stem Thermometer (Tech Instrumentation Inc., Elizabeth, CO, USA) while the whole system was maintained inside the incubator during LIPUS application.

3.2.4 Histology and histomorphometrical analysis:

The samples were fixed in 10% neutral buffered formalin (approx. 4% formaldehyde) (Sigma-Aldrich, Oakville, Ontario, Canada) after 5 days of culture then decalcified with CAL-EX(TM) II, Fisher Scientific, Ottawa, Ontario, Canada (formaldehyde 1.03 M/L, formic acid 2.56 M/L) for one week. Samples were

processed into paraffin blocks; sections of 5 μm thickness cut and stained using hematoxylin and eosin stain. Photomicrographs were taken using a Leica Fluorescent Digital Microscope with a (CCD) Digital camera (Leica, Wetzlar, Germany) and the image processing analyses were done using RS Image software (Version 1.73) (Photometrics, Roper Scientific, Inc, Tucson, AZ, USA). Cells of the odontoblastic layer in each section were counted in three random field/slices (0.02113 mm^2). Predentin thickness was measured in three random field/slices of each group. Tooth slices were allocated randomly to the ten groups during sectioning and we had a total of 540 slices with 54 per group for the histological evaluation (n= 54).

3.2.5 Real-Time Polymerase Chain Reaction:

Three to four slices of tissue cultured in the same well were grouped in order to have enough amount of mRNA. The slices from each well were snap frozen in liquid Nitrogen and then stored at -80° C. Afterward the tissue were pulverized while frozen using Mikro-Dismembrator S (Sartorius Mechatronics, Mississauga, ON, Canada) at 2500 rpm for one minute then the tissue powder was treated using TRIzol (Invitrogen, Burlington, ON, Canada). The mRNA was extracted using Qiagen RNeasy kit and QIAGEN RNase-Free DNase set for RNA extraction and purification. Omniscript RT Kit was used for reverse transcription of RNA into cDNA. Relative quantization of gene expression of the genes listed in Table 1 were performed using the relative standard curve method using Applied Biosystems 7500 Fast Real-Time PCR system using fluorescent SYBR

Green I dye based PCR (Integrated DNA Technologies, Inc. Coralville, IA, USA). Tooth slices were allocated randomly to the ten groups during sectioning and we had a total of 180 samples (3-4 slices per sample) for RT-PCR processing (n=18).

Table 3.1: Genes of interests primers and the housekeeping gene sequences used in the Q-PCR, these primers were designed using Primer Express® Software (Applied Biosystems, Canada).

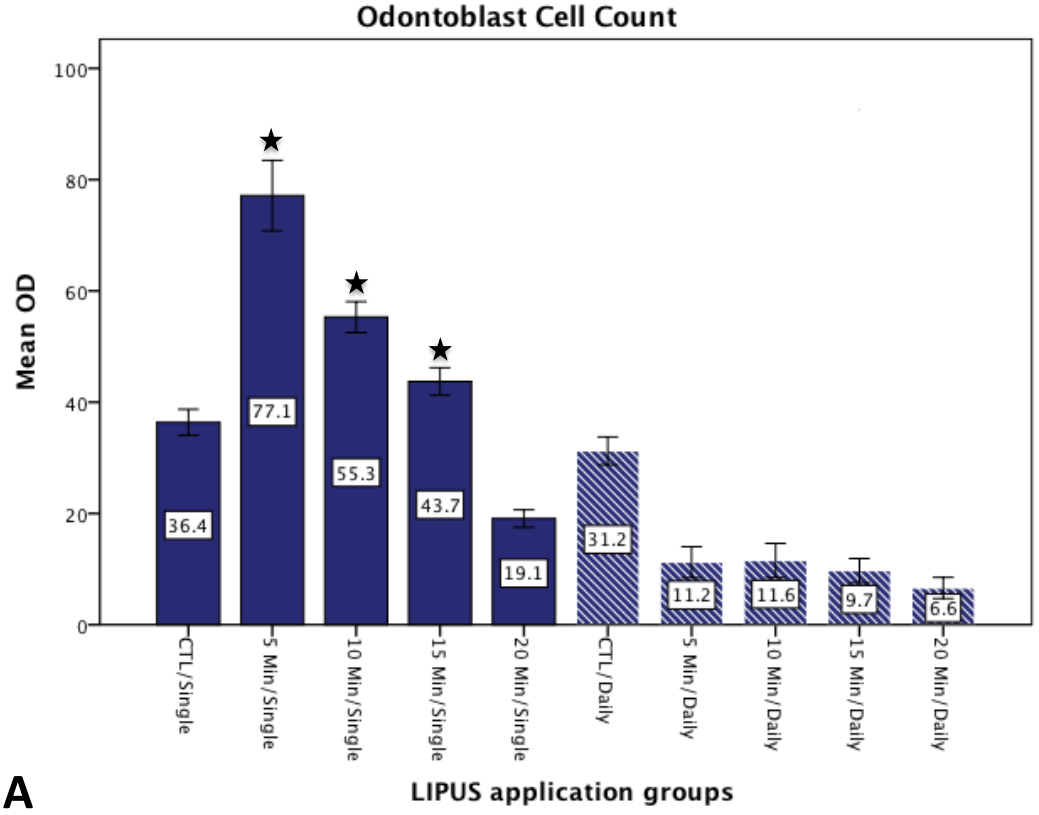
Primer	Sequence (5'-3')		Temp	bp
Dentin Matrix Protein1 (DMP1)	Sense	ATGGCCAGTTGAAAAACATTGA	58	36
	Antisense	CCCCAATGGGTTTGTTGTG	58	53
Dentin sialophosphoprotein (DSPP)	Sense	TGACTCAAAAGGAGCAGAAGATGAT	59	40
	Antisense	ATTTACCTTTGCCACTGTCTGATTT	58	36
Transforming Growth Factor β1 (TGF β1)	Sense	GGCCCTGCCCCTACATTT	58	61
	Antisense	CCGGGTTATGCTGGTTGTACA	59	52
Osteoprotegrin (OPG)	Sense	CGGCACATTGGACATGCTAA	59	50
	Antisense	TCCCACTTTCTTTCCCGGTAA	59	48
Receptor activator of NF-κB Ligand (RANKL)	Sense	TGGATGGCTCATGGTTAGATCTG	60	48
	Antisense	GCATTAATAGTGAGATGAGCAAAAGG	59	38
Collagen type I	Sense	CCTTTGCATTCATCTCTCAAACTTAG	59	38
	Antisense	GGTTGAATGCACTTTTGGTTTTT	58	35
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Sense	AAAAACCTGCCAAATATGATGACA	58	33
	Antisense	GCCCAGGATGCCCTTGA	59	65

3.2.6 Statistical Analysis:

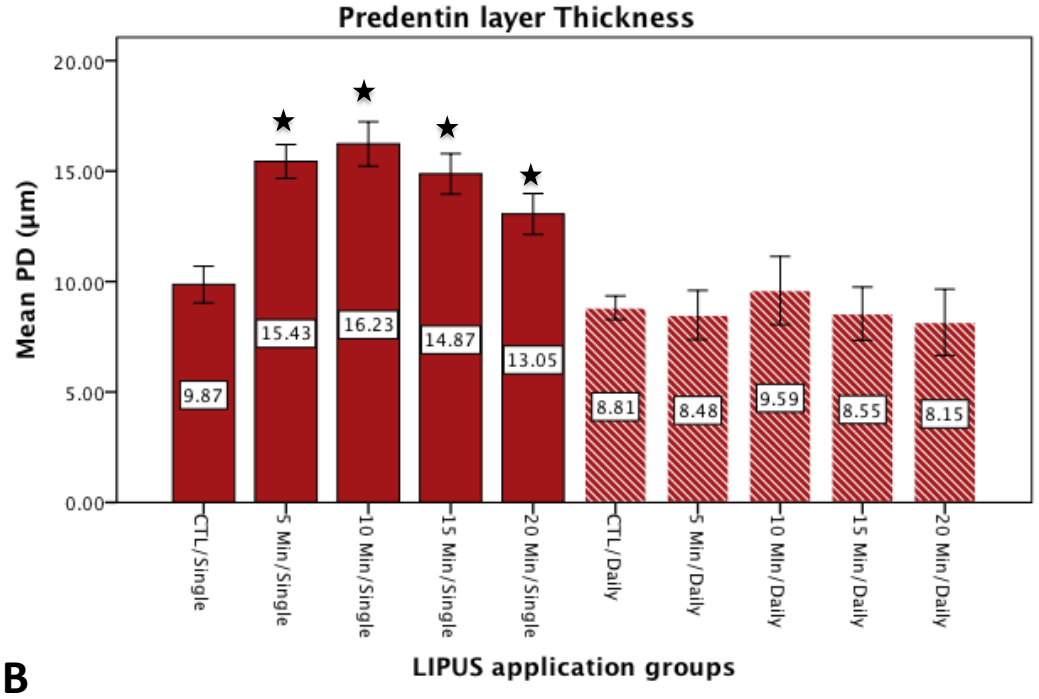
Data were collected and processed using SPSS 18.0. Means of the odontoblasts cell count and predentin thicknesses were compared using Kruskal-Wallis non-parametric test. Further multiple comparisons were carried out using Tamhane's test. Also, means of the expression of genes of interests were compared using Kruskal-Wallis non-parametric test. A p-value < 0.05 was considered statistically significant.

3.3 Results:

LIPUS application increased the temperature of the media but the change was very low, it had an average of 0.41˚C ± 0.36. The cell counts of the odontoblastic cell layers show that mean odontoblast cell numbers in the 5-, 10- and 15-minute LIPUS single application groups (77.13 ± 23.3, 55.3 ± 10.2 and 43.72 ± 8.9 respectively) were higher than all the other groups; moreover the differences among these three groups were statistically significant ($p < 0.01$). (Figure 3.1 A and Table 3.2) The odontoblast cell numbers in the 20-minute LIPUS/ single application group (19.09 ± 5.8) were lower than the control groups (control/single application = 36.39 ± 8.6, control/daily application = 31.24 ± 9.3) ($p < 0.01$) but higher than that of the daily application groups ($p < 0.01$). The daily application groups' cell numbers were lower than all the other groups including the control groups ($p < 0.01$); however the differences within the groups were not statistically significant. (Figure 3.1 A and Table 3.2)



A



B

Figure 3.1: A) Diagrams of the Mean Odontoblast cell count (OD) among the different control and LIPUS application groups per microscopic field (0.02113 mm^2).
B) Mean Predentin layer thickness. The hatched bars represent two standard deviations from the mean. [CTL: control, Min: Minutes of LIPUS application. ★: Statistically significant higher groups in the presented variable. Error bars present +/- 2 SD]

Evaluation of tissue and cellular morphology, demonstrated that the 5-, 10-, 15-minute LIPUS/ single application groups (Figure 3.2 B, C, and D) show a histological pattern that is similar to the control groups (Figure 3.2 and 3.3: A) in the abundance of darkly stained nuclei in the odontoblastic layer and lower density of the cells in the pulp core area. However, in the 20-minute LIPUS single application group, the cell density in both the odontoblastic cell layer and the pulp core was less than the control groups. (Figure 3.2 E) Daily application groups demonstrated poorly stained nuclei in the odontoblastic cell layer and loss of the integrity of cell and tissue architecture in that layer, and there was also a loss of darkly stained nuclei in the pulp core. (Figure 3.3 B, C, D and E)

From the histomorphometrical analysis, statistically significant differences among the LIPUS application groups in the odontoblast cell counts and the predentin layer thicknesses were observed. The predentin thickness was highest ($p < 0.01$) in the single application groups 10, 5 and 15 minutes (16.23 μm ± 3.7, 15.43 μm ± 2.8 and 14.87 μm ± 3.4 respectively), but they were not significantly different from each other ($p > 0.893$). The 20-minute/single application had mean predentin thickness (13.05 μm ± 3.4) larger than the control groups, which was statistically significant ($p < 0.01$), but not significantly different from the 15minute/single ($p = 0.252$) application group. The mean predentin thicknesses in all the four groups of LIPUS daily application (5 [8.48 μm ± 4.1], 10 [9.59 μm ± 5.7], 15 [8.54 μm ± 4.4] and 20 [8.15 μm ± 5.5] minutes) were not statistically significantly different from the control groups ($p > 0.895$), nor among the four groups ($p > 0.9$). (Figure 3.1 B and Table 3.2)

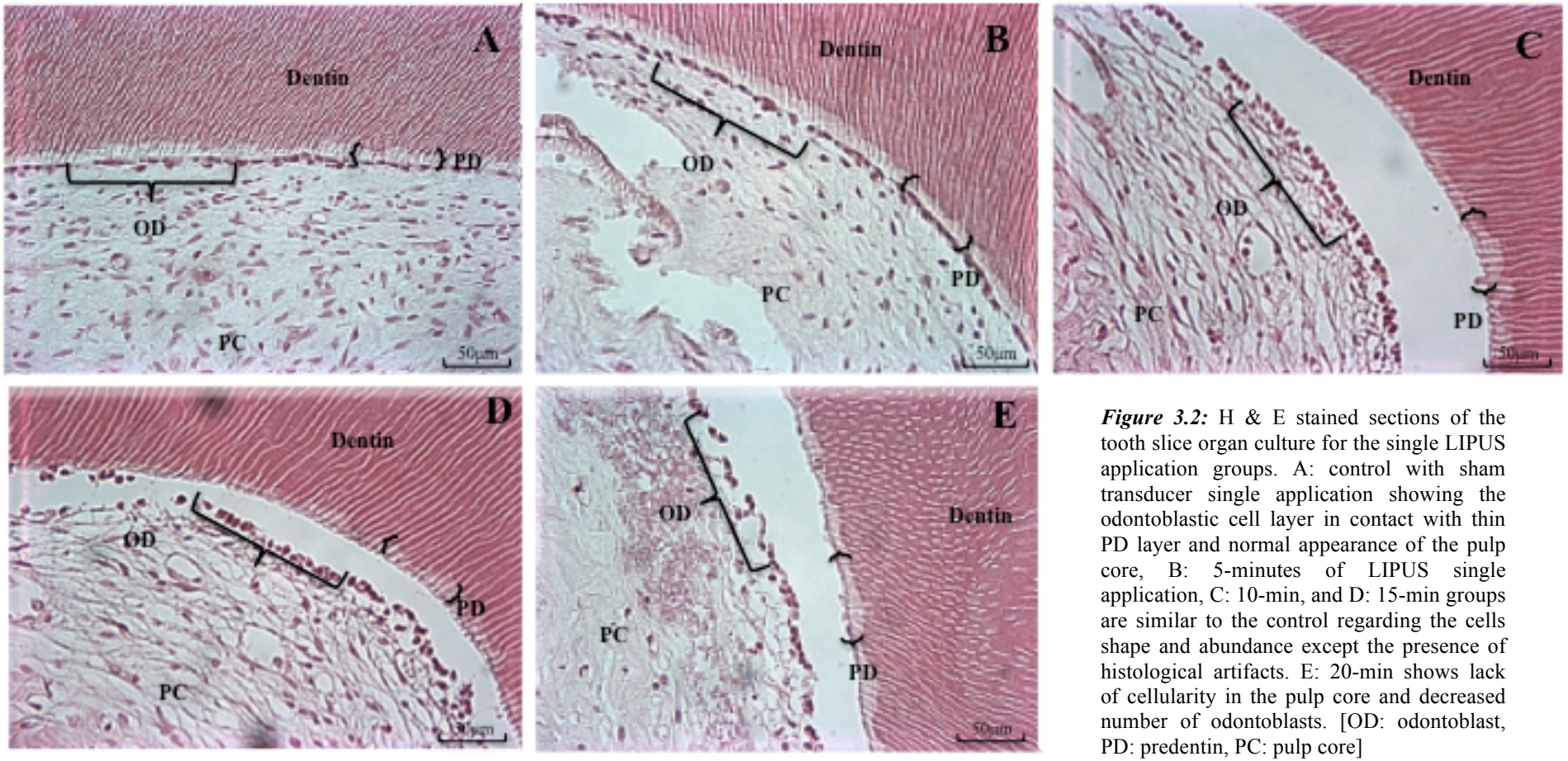


Figure 3.2: H & E stained sections of the tooth slice organ culture for the single LIPUS application groups. A: control with sham transducer single application showing the odontoblastic cell layer in contact with thin PD layer and normal appearance of the pulp core, B: 5-minutes of LIPUS single application, C: 10-min, and D: 15-min groups are similar to the control regarding the cells shape and abundance except the presence of histological artifacts. E: 20-min shows lack of cellularity in the pulp core and decreased number of odontoblasts. [OD: odontoblast, PD: predentin, PC: pulp core]

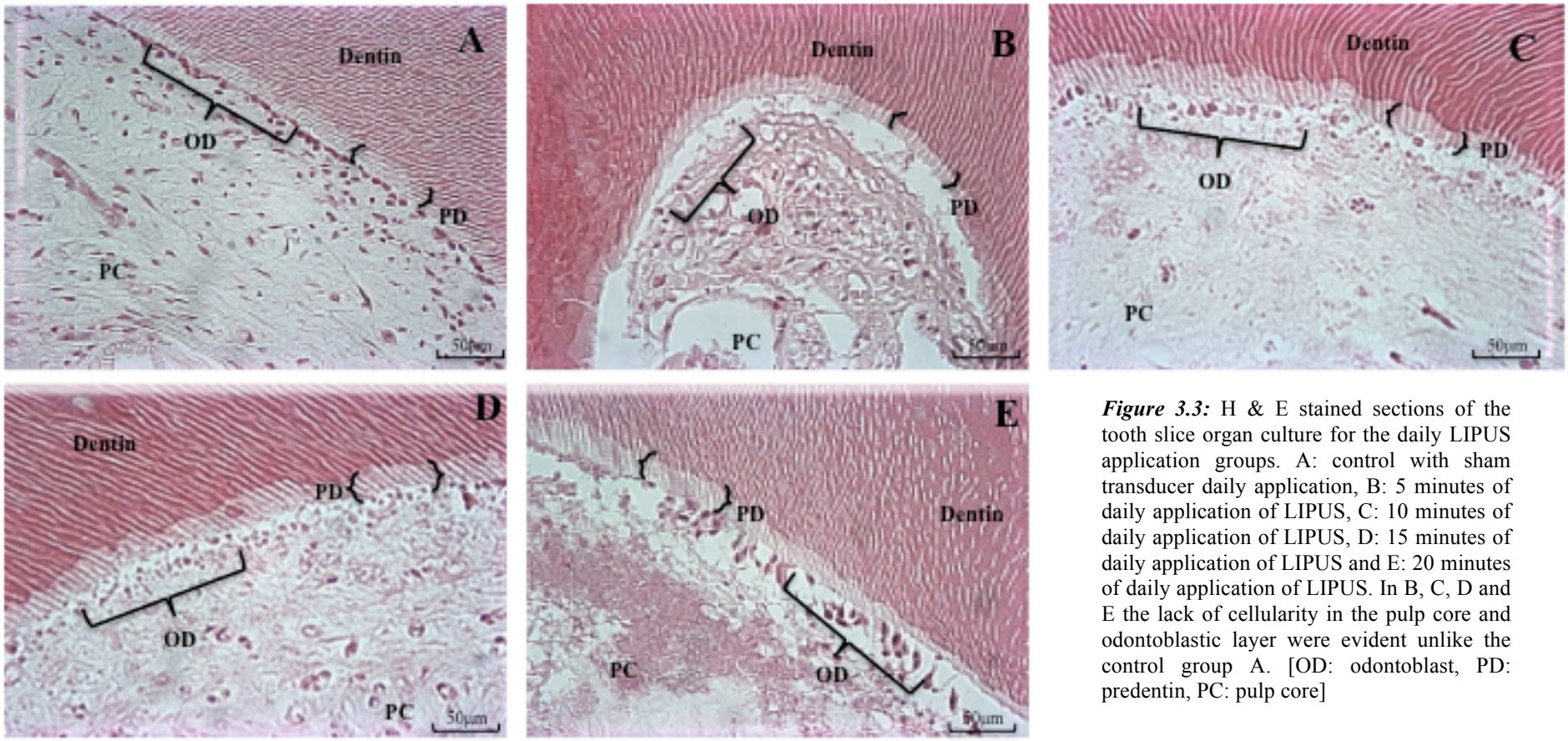


Figure 3.3: H & E stained sections of the tooth slice organ culture for the daily LIPUS application groups. A: control with sham transducer daily application, B: 5 minutes of daily application of LIPUS, C: 10 minutes of daily application of LIPUS, D: 15 minutes of daily application of LIPUS and E: 20 minutes of daily application of LIPUS. In B, C, D and E the lack of cellularity in the pulp core and odontoblastic layer were evident unlike the control group A. [OD: odontoblast, PD: predentin, PC: pulp core]

Table 3.2: The results of multiple comparisons (Tamhane's test) between groups for the variables predentin (PD) and odontoblast count (OD) [CTL: control, Dif: mean difference, SE: standard error, P: p-value *: Statistically significance].

		CTL/Single		LIPUS 5/ Single		LIPUS 10/ Single		LIPUS 15/ Single		LIPUS 20/ Single		CTL/Daily		LIPUS 5/Daily		LIPUS 10/Daily		LIPUS 15/Daily		LIPUS 20/Daily	
		Dif	P	Dif	P	Dif	P	Dif	P	Dif	P	Dif	P	Dif	P	Dif	P	Dif	P	Dif	P
CTL /Single	PD			-5.5	<0.01*	-6.4	<0.01*	-5.0	<0.01*	-3.2	<0.01*	1.1	0.8	1.4	0.9	0.3	1.0	1.3	0.9	1.7	0.9
	OD			-40.7	<0.01*	-18.9	<0.01*	-7.3	<0.01*	17.3	<0.01*	5.2	0.14	25.2	<0.01*	24.8	<0.01*	26.7	<0.01*	29.8	<0.01*
LIPUS 5 /Single	PD	5.5	<0.01*			-0.8	1.0	0.6	1	2.4	<0.01*	6.6	<0.01*	6.9	<0.01*	5.8	<0.01*	6.9	<0.01*	7.3	<0.01*
	OD	40.7	<0.01*			21.8	<0.01*	33.4	<0.01*	58	<0.01*	45.9	<0.01*	65.9	<0.01*	65.6	<0.01*	67.4	<0.01*	70.5	<0.01*
LIPUS 10 /Single	PD	6.4	<0.01*	0.8	1.0			1.4	0.9	3.2	<0.01*	7.4	<0.01*	7.7	<0.01*	6.6	<0.01*	7.7	<0.01*	8.1	<0.01*
	OD	18.9	<0.01*	-21.8	<0.01*			11.6	<0.01*	36.2	<0.01*	24.1	<0.01*	44.1	<0.01*	43.7	<0.01*	45.6	<0.01*	48.7	<0.01*
LIPUS 15 /Single	PD	5.0	<0.01*	-0.6	1	-1.4	0.9			1.8	0.3	6.1	<0.01*	6.4	<0.01*	5.3	<0.01*	6.3	<0.01*	6.7	<0.01*
	OD	7.3	<0.01*	-33.4	<0.01*	-11.6	<0.01*			24.6	<0.01*	12.5	<0.01*	32.5	<0.01*	32.2	<0.01*	34	<0.01*	37.1	<0.01*
LIPUS 20 /Single	PD	3.2	<0.01*	-2.4	<0.01*	-3.2	<0.01*	-1.8	0.3			4.2	<0.01*	4.6	<0.01*	3.5	<0.01*	4.5	<0.01*	4.9	<0.01*
	OD	-17.3	<0.01*	-58	<0.01*	-36.2	<0.01*	-24.6	<0.01*			-12.2	<0.01*	7.9	<0.01*	7.5	<0.01*	9.4	<0.01*	12.5	<0.01*
CTL /Daily	PD	-1.1	0.8	-6.6	<0.01*	-7.4	<0.01*	-6.1	<0.01*	-4.2	<0.01*			0.3	1.0	-0.8	1.0	0.3	1.0	0.7	1.0
	OD	-5.2	0.14	-45.9	<0.01*	-24.1	<0.01*	-12.5	<0.01*	12.2	<0.01*			20	<0.01*	19.7	<0.01*	21.5	<0.01*	24.6	<0.01*

RT-PCR demonstrated that there were no statistically significant differences among the control and LIPUS application groups in the expression of any of the genes of interest. (Table 3.1) The DMP1, TGF β1 and collagen I were expressed in all groups in low amounts that were detectable but the differences among the groups were not statistically significant ($p > 0.168$). (Figure 3.4) Interestingly, dentin sialophosphoprotein (DSPP), RANKL and osteoprotegerin (OPG) were expressed at very low levels in all groups. (Figure 3.5) In addition the two control groups did not have any statistical significant differences in any of the tested variables. (Table 3.2)

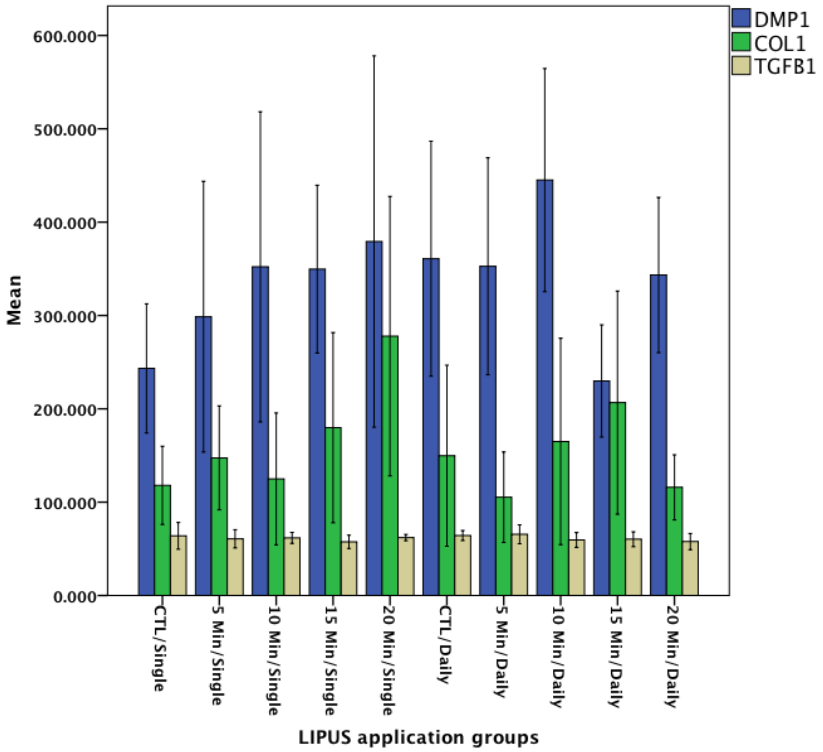


Figure 3.4: Diagram showing the expression of DMP1, TGF β1 and collagen I among the ten groups.

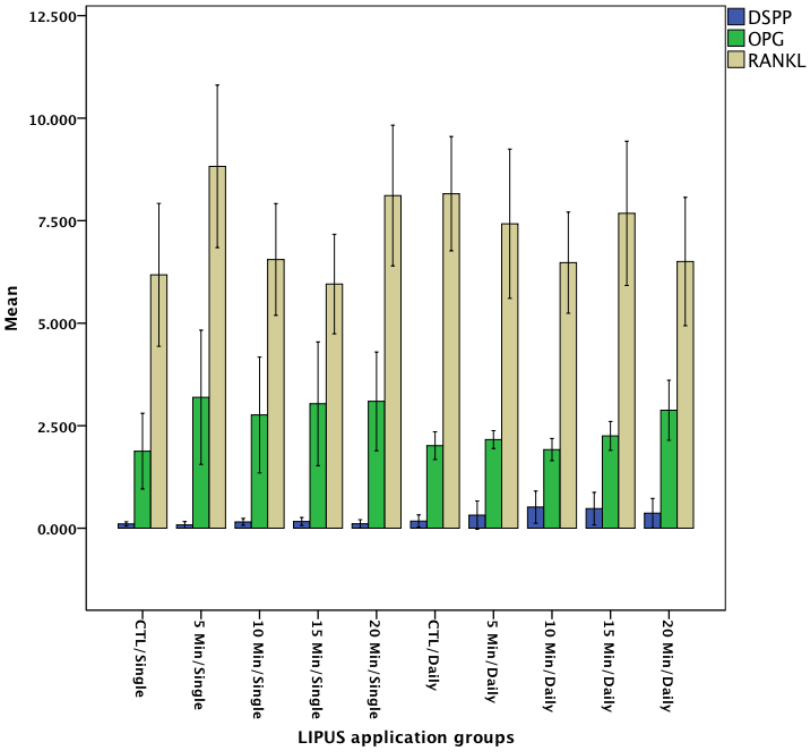


Figure 3.5: Diagram showing the expression of DSPP, RANKL and OPG among the ten groups: These genes were expressed in very small amounts especially DSPP which was barely detectable in some samples and not expressed at all in others.

3.4 Discussion:

Despite the multiple attempts to identify the biological effects of therapeutic ultrasound, the intracellular mechanism and physical interaction through which it affects living tissue remains unknown. When the acoustic waves pass through the tissue, the energy is absorbed at a rate proportional to the density of the tissue. This differential absorption may play a critical role in targeting the cells present inside and around the hard tissue (e.g. callus in bone fracture and pulp or PDL of the tooth). In this study we tested the hypothesis of whether LIPUS has a stimulatory effect on the dentin-pulp complex *in vitro*. Stimulating the secretion of extracellular matrix by the odontoblast cells would result in narrowing or even sclerosing of the dentinal tubules. This will result in decreased permeability of the dentin layer which can be more resistant to bacterial or toxin penetration from cariogenic bacteria; also a denser dentinal layer may be more resistant to dentin resorption.

We investigated the effects of LIPUS on the dentin-pulp complex in this *in-vitro* model by examining its impact on odontoblast cell number and secretory activity manifested by the predentin layer thickness. Furthermore, we focused on the alteration in expression of the primary dentin matrix component (collagen I), extracellular matrix mineralization initiation and propagation aiding non-collagenous proteins (DMP 1 and DSPP), stimulating growth factors (TGF ß1) and odontoclasts regulating factors (RANKL and OPG) in the dentin-pulp complex. Thermal effects can occur when using intensities as high as 1–3W/cm^2, which can cause considerable heating of tissues but in studies using LIPUS (20–

50mW/cm^2) heating effect was reported to be below 1˚C.[9] Likewise in our study the average increase of the media temperature was 0.45˚C ± 0.23.

In the histomorphometrical analysis findings, higher odontoblast cell numbers were only evident in the 5-, 10- and 15-minute LIPUS single application. Previous research demonstrated the same effect of LIPUS application with fibroblasts, [10] cementoblasts,[11] osteoblasts[12] and chondrocytes.[13] Because odontoblasts are postmitotic cells;[14] the higher cell number in the histomorphometrical analysis can be attributed to fewer cells going through apoptosis in this tissue culture model. Alternatively it can be attributed to differentiation of functional odontoblasts from their potential precursors which have been found in localized perivascular niches in the adult human pulp (dental pulp stem cells [DPSCs]).[15] Reports of differentiation of pulp mesenchymal stem cells into odontoblasts or odontoblast-like cells *in vitro* [16] and *in vivo*[17] bring this possibility into consideration. LIPUS applications for 5, 10, and 15 minutes may led to minimizing apoptosis of the odontoblastic layer. This could be in agreement with a previous study that showed that LIPUS has an anti-apoptosis effect.[18] Another indirect mechanism can be a transient[19] increase in the expression DMP1 and TGF-ß1 that have been previously reported to be promoting factors of odontoblast differentiation from undifferentiated mesenchymal cells. [20] LIPUS has been shown to induce the differentiation process of different cell lines like the human gingival fibroblasts[21] and cementoblasts.[8]

The deterioration and loss of normal histological architecture in the groups of daily LIPUS application was noticeable. This effect has been reported in other

studies with short-term application to odontoblast cell lines,[22,23] but Suzuki *et al* reported that LIPUS did not affect the cell viability of rat osteoblasts *in vitro* when applied daily for 20 min with an intensity of 30 mW/cm^2 (1.5 MHz) for 14 days.[24] Our findings demonstrate that daily LIPUS application may not be biologically tolerable by the human pulp cell population and may cause faster apoptosis *in vitro*. But in this model cells are being tested in a tissue matrix (3D rather than 2D) and therefore they may behave very differently than cells in monolayer culture. Cells in contact with tissue and in the absence of vascular system may modify the effects of mechanical stimulation that may be too much by the time we get to 20 min applications. In contrast 20 min of LIPUS application were reported to be optimal *in vivo* [ref?] which is totally a different environment. Future studies will be aimed at identifying the optimum LIPUS treatment that may enhance odontoblastic viability and differentiation in human tooth slice organ culture.

Evaluation of predentin thickness variation shows some evidence of the stimulatory effect of LIPUS on the secretory function of the odontoblasts. This could be attributed to the slight increase in DMP1 and Col I genes, although it was not statistically significant. Also, this could be explained by the increase of DSPP after one single LIPUS application (although this increase is not statistically significant). Because our study used random sample allocation and examiner blinding, potential concerns over the short period of culture and the normal variation in predentin thickness in human premolars [25] were minimized.

The investigation of gene expression attempted to explore further the effect of LIPUS on the dentin-pulp complex at the molecular level. From the RT-

PCR results we found that TGF-β1 and collagen-I were detectable in all groups but there was no statistically significant difference among the groups. In another study it was reported that low frequency ultrasound (30kHz) resulted in an increase in the expression of collagen type I, osteopontin (OPN), TGF β1 and the heat shock protein (hsp) 70; but this was in a short-term (4 and 24 hours post-exposure) evaluation of an odontoblast cell line culture.[23] TGF-β1 was of interest in this study because it has been shown to influence the behaviour of dental pulp cells.[4] Specifically, it stimulates the main events of human dental pulp repair including cell proliferation, cell migration, and type I collagen synthesis.[26] Also, TGF-β1 was found to stimulate matrix secretion and initiate odontoblast cytodifferentiation *in vitro* and *in vivo.* [4] LIPUS was reported to stimulate chondrogenic differentiation of rabbit mesenchymal stem cells cultured on alginate beads without TGF-β treatment.[27] In the present *in vitro* study if any upregulation or difference in the gene expression existed due to LIPUS application, it may have been concealed in the PCR results by two possibilities. The first one is that the upregulation wasn't at a detectable level due to the small difference between the groups and moderately large standard deviation (relative to the mean). The second possibility was that the LIPUS effect was only transient and time dependent[28] and therefore not identified in our experiment due to the relatively long latent period between the LIPUS application and the harvest time.

In regard to odontoblast-related genes, DMP1 is a non-collagenous protein that has a regulatory effect on development and differentiation of the odontoblasts, the initiation of hydroxyapatite nucleation and regulates the expression of osteocalcin, alkaline phosphatase, and DSPP.[29] Another non-

collagenous protein that is specific for the odontoblasts is the DSPP that cleaves into dentin sialoprotein (DSP) and dentin phospophyren (DPP), where the DPP interacts specifically with collagen and initiates hydroxyapatite crystals and controls the rate of crystal growth.[30] In the present study, RT-PCR results showed that DMP1 was expressed in all groups with no statistically significant difference between the groups. On the other hand, the expression of DSPP was barely detectable in all the test and control groups that may be attributed to the cleavage process of DSPP into two non-collagenous proteins (DSP and DPP) that may occur anytime between the moment of mRNA synthesis and the tissue harvest process. Future research may aim at detecting the expression of these proteins by immunohistochemistry.

The OPG/RANK/RANKL pathway controlling the osteoclastogenesis and odontoclastogenesis was found to exist in physiologic root resorption in deciduous teeth. [31] Lossdorfer *et al* suggested that odontoclasts had an autocrine-paracrine role. [32] Our study is limited in representing an autocrine-paracrine role due to lack of circulation and due to the limited time in tissue culture when compared to *in vivo* studies. Low intensity ultrasound at 1.5 MHz/30 mW/cm^2 was found to increase the expression of RANKL and decrease OPG expression by SaOS-2.[33] On the other hand, a higher power of ultrasound at 62.5 or 125 mW/cm^2 markedly inhibited RANKL plus M-CSF-induced osteoclastic differentiation from bone marrow stromal cells.[34] From the RT-PCR results in the present *in vitro* study, RANKL and OPG were expressed in very low amounts in all groups with no significant difference among the groups. This may indicate that

dentin-pulp complex may not be the optimum model for studying odontoclastogenesis and supports that PDL is the key player in this process.

3.5 Conclusion:

LIPUS demonstrated a stimulatory effect on human tooth slice organ culture at the histological level when applied once but had a deteriorating effect when applied daily. Five days of culture was a long time for a human tooth slice organ culture but the model was reliable and reproducible. Further investigation of the LIPUS effect on this *in vitro* model is needed to demonstrate its actual effects at the molecular level.

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Chapter 4:

Short-term effect of Low Intensity Pulsed Ultrasound on a human tooth slice organ culture

4.1 Introduction:

Low Intensity Pulsed Ultrasound (LIPUS) is an acoustic vibration with frequencies above the limit of human hearing that can transmit into the body as high frequency mechanical waves. These mechanical waves' energy is absorbed at a rate proportional to the acoustic density of the tissues in which it passes through. The micromechanical strains produced by the pressure waves while passing through the biological tissues are assumed to initiate biochemical events that affect hard and soft tissue activity. This form of ultrasound has been already established as a therapeutic form of ultrasound that can enhance healing of bone fracture.[1]

Due to the non-invasive nature of LIPUS, the interest in its application has been extended into studying its effect on dental and periodontal tissues. It was reported that low- and high-intensity ultrasound increases the expression of alkaline phosphatase (ALP) by cementoblasts, with no effect on cell proliferation. [2] Also it was found that LIPUS enhances ALP activity, collagen synthesis, and Runx2 expression by human periodontal ligament cells *in vitro*, which provide important insight into the promotion of early cementoblastic differentiation of immature cementoblasts.[3]

Dentin pulp complex is the principal tissue in the process of hard tooth structure initial development and reparative mechanism when an assault is introduced to the tooth by forming reparative dentin. Like any specialized cell line; attempts to culture odontoblasts *in vitro* have been done. However, it has

been shown that their contact with the extracellular dentin matrix is crucial for maintaining their phenotypic morphology and secretory activity.[4]

A human tooth organ culture model for the dentin-pulp complex has been reported in which cell viability can be maintained for different periods and up to 14 days. This model is useful in testing the cytotoxicity of dental restorative materials and the stimulatory effect of growth factors on the dentin-pulp complex.[5] Moreover, dentinogenesis in rat incisors [6] and the effect of transforming growth factor-β (TGF-β) isoforms on dentinogenesis and extracellular matrix secretion by odontoblasts were studied *in vitro* using this model. They found that TGF-β1 and TGF-β3 can stimulate secretion of extracellular matrix by odontoblasts and that TGF- β3 may have inductive effects on pulpal cells. [7] Hence LIPUS was found to increase the expression of TGF-β1 [8], we hypothesizes that LIPUS application to the dentin-pulp complex would enhance dental tissue formation.

Tumor necrosis factor receptors-ligand family osteoprotegrin (OPG) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) balance has been reported to determine the osteoclastogenesis and odontoclastogenesis. [9] Also it was found that this mechanism is crucial in root resorption process and the number of odontoclasts.[10-12]

In this experiment we investigated the effect of LIPUS on a 3-D tissue culture of the dentin-pulp complex *in vitro* to demonstrate any effects on the cellular and molecular levels in the dentin-pulp complex. This *in vitro* model can

maintain a close relationship between the odontoblastic cell layer and the dentin matrix. Also, it provides the flexibility of investigation and control of the study design in an *in vitro* environment to determine the expression of extracellular matrix proteins genes (Col I, DMP1, DSPP), growth factor (TGF ß1) and odontoclastogenesis controlling cytokines (RANKL, OPG).

4.2 Materials and Methods:

4.2.1 Samples and sample collection:

Healthy permanent premolars were obtained from 18 adolescent orthodontic patients with a mean age of 12y-8m ± (14 m) who needed extraction for their orthodontic treatment. Inclusion criteria for these premolars include teeth that are sound (no fillings or caries) and have an open apex on the panoramic x-ray film. Sixty-three premolars were obtained and 8-11 slices were obtained per tooth. The slices were allocated randomly to five groups according to LIPUS application (5, 10, 15 and 20 minutes [for one application]) or control group (no treatment). A control group received a sham transducer that didn't emit LIPUS. Ethical approval for collecting the samples was obtained from the Health Research Ethics Board at the University of Alberta.

4.2.2 Tooth slice organ culture:

Extracted teeth were placed in a sterile washing medium consisting of DMEM containing antibiotic/antimycotic (1000 units/mL penicillin G sodium, 10µg/mL streptomycin sulphate and 25µg/ml amphotericin B). The teeth were

sectioned transversely into 600 μm-thick sections with a 0.006" diamond wafer saw (ISOMET® Wafering Blade, Series 15HC, 3" x 0.006" x 1/2") (Buehler, Whiteby, Ontario, Canada) and cooled with sterile PBS. Tooth slices were immediately placed in sterile washing medium before culture and washed several times at 37°C immediately after cutting, and then transferred into individual wells of a plastic 6-well plate containing 4 ml of culture media. The culture medium contained DMEM, vitamin C (0.15 mg/ml), 10% heat inactivated fetal calf serum, L-glutamine (200 mM), and 1% penicillin/streptomycin solution. Tooth slices were cultured at 37°C in an atmosphere of 5% CO_2 in air, in a humidified incubator, and the medium was changed after 24 hours. Two hours after the medium change we applied LIPUS to the samples and harvested them 24 hours after the application time.

4.2.3 Ultrasound application and calibration:

Custom-built ultrasound devices that provided adjustable output parameters and long-term operation stability were used (Smilesonica Inc., Edmonton, AB, Canada). The devices were set to generate ultrasound pulses with a repetition rate of 1 KHz. Each pulse has duration of 200 microseconds and a pulse frequency of 1.5 MHz. For each device, the ultrasound transducer has an emitting surface area of 3.9 cm^2 and it generates a temporal average ultrasound power of 120mW (or a temporal average ultrasound intensity of 30 mW/cm^2). The transducer was applied sequentially below each of the 6-well plates using high viscosity gel (National Therapy Products Inc., Woodbridge, ON, Canada) in between as a

coupling media inside the incubator. The five groups received one LIPUS application for the duration assigned to the group (5, 10, 15 and 20 minutes) or no LIPUS (control). The amount of signal attenuation after passing through the plate material was measured in the lab and it was found to be 4% of the total output power. At the beginning and at the end of the experiments the ultrasound devices were inspected for consistency of electrical waveforms (1 KHz modulation, 200 microseconds pulse duration, and 1.5MHz pulse frequency) using TDS1012C-EDU digital oscilloscope (Tektronix, Canada), and calibrated for ultrasound intensity of 30mW/cm2 using an ultrasound power-meter (model UPM-DT-1AV from Ohmic Instruments, Easton, MD, USA). The calibrations at the beginning and at the end of the experiment confirmed that the ultrasound devices provided stable power output and maintained the desired parameters during the experiment. Culture media were monitored for thermal changes due to the application of LIPUS using TL1A Series High Accuracy Digital Stem Thermometer (Tech Instrumentation Inc., Elizabeth, CO, USA) while the whole system was maintained inside the incubator during LIPUS application.

4.2.4 Histology and histomorphometrical analysis:

The samples were fixed in 10% neutral buffered formalin (approx. 4% formaldehyde) (Sigma-Aldrich, Oakville, Ontario, Canada) 24 hours after LIPUS application, then decalcified with CAL-EX(TM) II, Fisher Scientific, Ottawa, Ontario, Canada (formaldehyde 1.03 M/L, formic acid 2.56 M/L) for one week. Samples were processed into paraffin blocks; sections of 4 mm thickness were cut

and stained using hematoxylin and eosin stain. Photomicrographs were taken using a Leica Fluorescent Digital Microscope with a (CCD) Digital camera (Leica, Wetzlar, Germany) and the image processing analyses were done using RS Image software (Version 1.73) (Photometrics, Roper Scientific, Inc, Tucson, AZ, USA). Cells of the odontoblastic layer in each section were counted in three random field/slices (0.02113 mm^2). Predentin thickness was measured in three random field/slices of each group. Tooth slices were allocated randomly to the five groups during sectioning, and we had a total of 125 slices with 25 per group for the histological evaluation (n= 25).

4.2.5 Real-Time Polymerase Chain Reaction:

Three to four slices of tissue cultured in the same well were grouped in order to have enough mRNA. The slices from each well were snap frozen in liquid Nitrogen and then stored at -80° C. Afterward, the tissue was pulverized while frozen using Mikro-Dismembrator S (Sartorius Mechatronics, Mississauga, ON, Canada) at 2500 rpm for one minute then the tissue powder was treated using TRIzol (Invitrogen, Burlington, ON, Canada). The mRNA was extracted using Qiagen RNeasy kit and QIAGEN RNase-Free DNase set for RNA extraction and purification. RNA quality and integrity were measured by performing a Eukaryote total RNA Nano assay using Agilent 2100 Bioanalyzer (Agilent Technologies Canada Inc., Mississauga, ON, Canada). An Omniscript RT Kit was used for reverse transcription of RNA into cDNA. Relative quantization of gene expression of the genes listed in Table 4.1 were performed using the relative

standard curve method using Applied Biosystems 7500 Fast Real-Time PCR system using fluorescent SYBR Green I dye based PCR (Integrated DNA Technologies, Inc. Coralville, IA, USA). Tooth slices were allocated randomly to the five groups during sectioning, and we had a total of 150 samples for RT-PCR processing (n=30).

4.2.6 Statistical Analysis:

Data were collected and processed using SPSS 18.0. Means of the odontoblasts cell count and predentin thicknesses were compared using MANOVA test. Also, means of the expression of genes of interests were compared using MANOVA. A P-value of < 0.05 was considered statistically significant. Further multiple comparisons were carried out using Tamhane's test.

4.3 Results:

LIPUS application increased the temperature of the media but the change was very low, it had an average of 0.41˚C ± 0.36. Histomorphometrical analysis showed no statistical significant difference among the five groups in measured histomorphometric analysis variables, odontoblast cell count, $P = 0.074$ (Figure 4.1) and predentin thickness, $P = 0.33$ (Figure 4.2) by running MANOVA (Table 4.2).

Table 4.1: Genes of interests primers and the housekeeping gene sequences used in the Q-PCR, these primers were designed using Primer Express® Software (Applied Biosystems, Canada).

Primer	Sequence (5'-3')		Temp	bp
Dentin Matrix Protein1 (DMP1)	Sense	ATGGCCAGTTGAAAAACATTGA	58	36
	Antisense	CCCCAATGGGTTTGTTGTG	58	53
Dentin sialophosphoprotein (DSPP)	Sense	TGACTCAAAAGGAGCAGAAGATGAT	59	40
	Antisense	ATTTACCTTTGCCACTGTCTGATTT	58	36
Transforming Growth Factor β1 (TGF β1)	Sense	GGCCCTGCCCCTACATTT	58	61
	Antisense	CCGGGTTATGCTGGTTGTACA	59	52
Osteoprotegrin (OPG)	Sense	CGGCACATTGGACATGCTAA	59	50
	Antisense	TCCCACTTTCTTTCCCGGTAA	59	48
Receptor activator of NF-κB Ligand (RANKL)	Sense	TGGATGGCTCATGGTTAGATCTG	60	48
	Antisense	GCATTAATAGTGAGATGAGCAAAAGG	59	38
Collagen type I	Sense	CCTTTGCATTCATCTCTCAAACTTAG	59	38
	Antisense	GGTTGAATGCACTTTTGGTTTTT	58	35
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Sense	AAAAACCTGCCAAATATGATGACA	58	33
	Antisense	GCCCAGGATGCCCTTGA	59	65

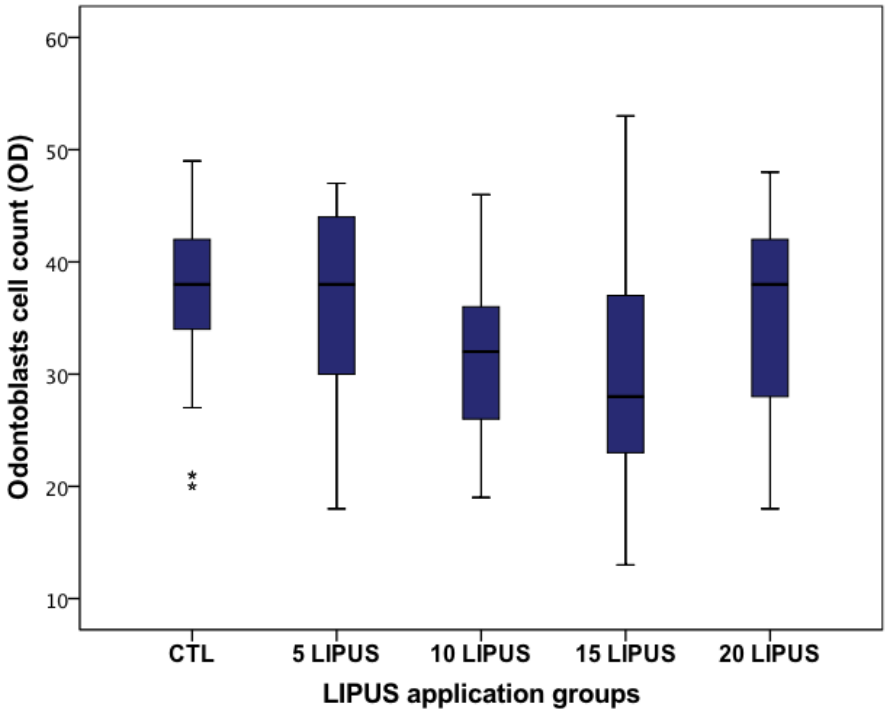

Figure 4.1: Box plot of the Odontoblast cell count among the five groups showing very small variation and few outliers in the control group. (CTL: Control, # LIPUS: # minutes of LIPUS application)

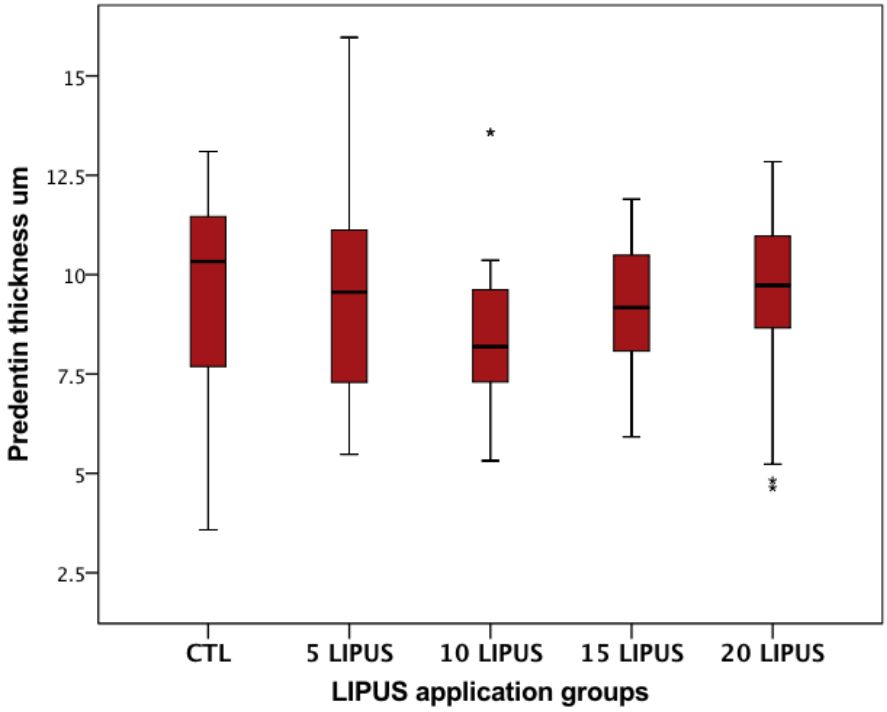

Figure 4.2: Box plot of the predentin thickness (µm) among the five groups showing very small variation and few outliers in the ten- (10 LIPUS) and twenty- (20 LIPUS) minute application groups. (CTL: Control, # LIPUS: # minutes of LIPUS application)

Table 4.2: Statistical analysis results (MANOVA) for all the measured dependent variables (OD: odontoblast cell count. PD: predentin thickness. Col 1: collagen type I. DMP1: dentin matrix protein. DSPP: dentin sialophosphoprotein. TGF ß1: transforming growth factor ß1. logOPG: log transformation of osteoprotegrin expression results)

	Histology		RT-PCR					
	OD	PD	Col 1	DMP1	DSPP	RANKL	TGF ß1	logOPG
MANOVA P-value	0.074	0.33	<0.01*	<0.01*	<0.01*	<0.01*	0.8	<0.01*

Evaluation of tissue and cellular morphology, the control and the four LIPUS application groups showed almost the same histological pattern and viability. (Figure 3: A-E) The odontoblastic cell layer was well defined with a somewhat cuboidal cell shape different from the traditional and normal *in-vivo* representation, which has the pseudo-stratified columnar architecture. The odontoblast cell layer shows close adaptation to the predentin layer that has a fairly average and uniform thickness in the five groups. The pulp core has light to medium cellular density with normal shaped cells and darkly stained nuclei, otherwise no significant observations. (Figure 3)

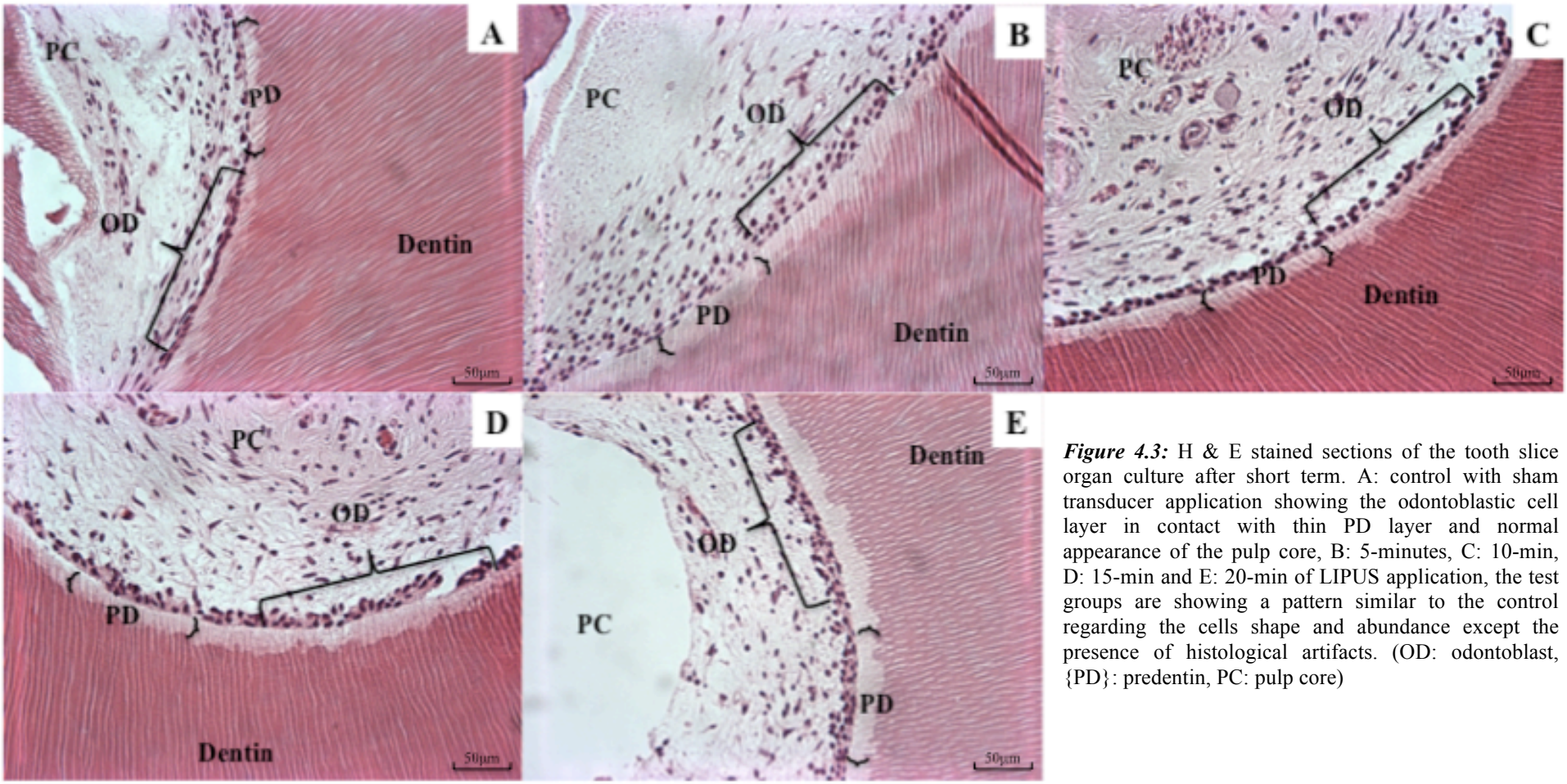

Figure 4.3: H & E stained sections of the tooth slice organ culture after short term. A: control with sham transducer application showing the odontoblastic cell layer in contact with thin PD layer and normal appearance of the pulp core, B: 5-minutes, C: 10-min, D: 15-min and E: 20-min of LIPUS application, the test groups are showing a pattern similar to the control regarding the cells shape and abundance except the presence of histological artifacts. (OD: odontoblast, {PD}: predentin, PC: pulp core)

RT-PCR demonstrated that there was a statistically significant difference among the control and LIPUS application groups in the expression of all the expressed genes ($P < 0.01$). (Table 2) TGF β1 ($P = 0.8$) was the only gene that did not express in any of the five groups. (Figure 4) Although RANKL and OPG expression showed statistically significant differences, their expression was very low relative to the housekeeping gene GAPDH, but the statistical significance resulted from the fact that OPG was only expressed in the 15-minute LIPUS application group ($P < 0.01$) {We used the log-transformed values of the OPG gene expression results because the raw data didn't meet the assumptions for carrying out MANOVA- equal variance assumption}. (Figure 5) Furthermore, RANKL was expressed in all groups in very low amounts, but was highest in expression in the 15-minute LIPUS application group too ($P < 0.01$). (Figure 6)

DSPP was expressed in very low amounts in all groups without any significant difference between them except the 20-minute LIPUS application group, which had lower expression of DSPP than all the test and control groups, and that difference was statistically significant ($P < 0.01$). (Table 3, Figure 7) Another non-collagenous protein gene expression was investigated, DMP1 which was expressed in larger amounts than DSPP. DMP1 was expressed in the 10-minute LIPUS application group three times higher than the expression in the other groups ($P < 0.01$). (Table 3, Figure 8)

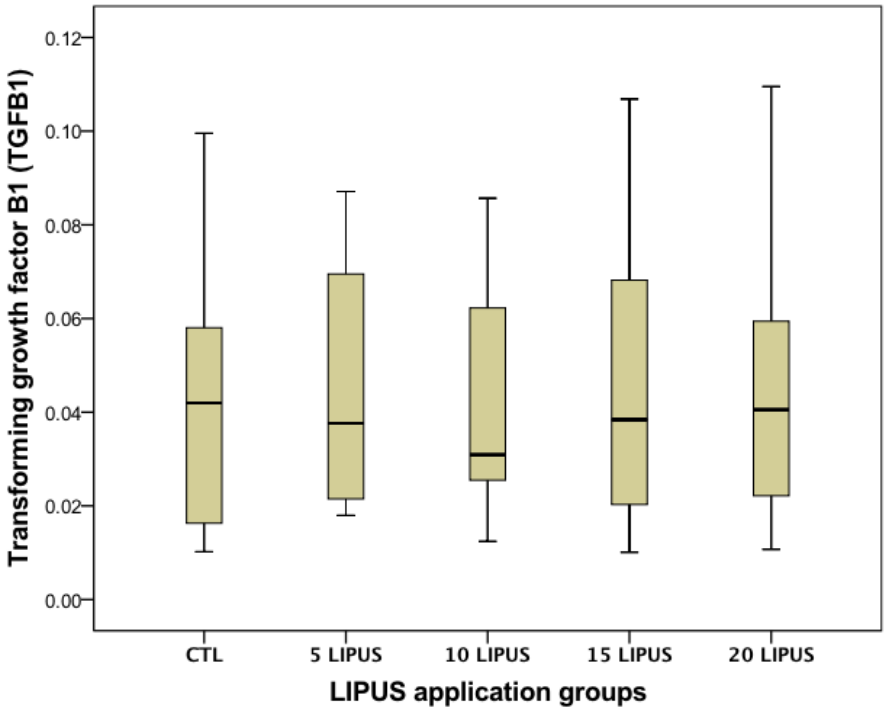

Figure 4.4: Box plot of the expression of TGF β1 among the five groups showing very small expression of this gene. (CTL: Control, # LIPUS: # minutes of LIPUS application)

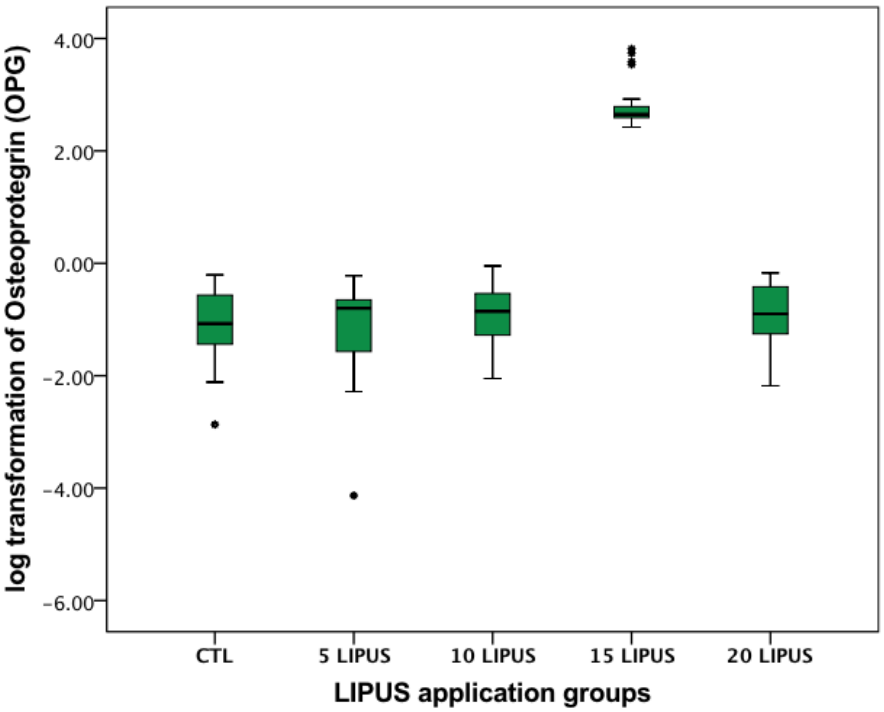

Figure 4.5: Box plot of the log transformation of the expression results of OPG among the five groups showing very small expression of this gene. (CTL: Control, # LIPUS: # minutes of LIPUS application)

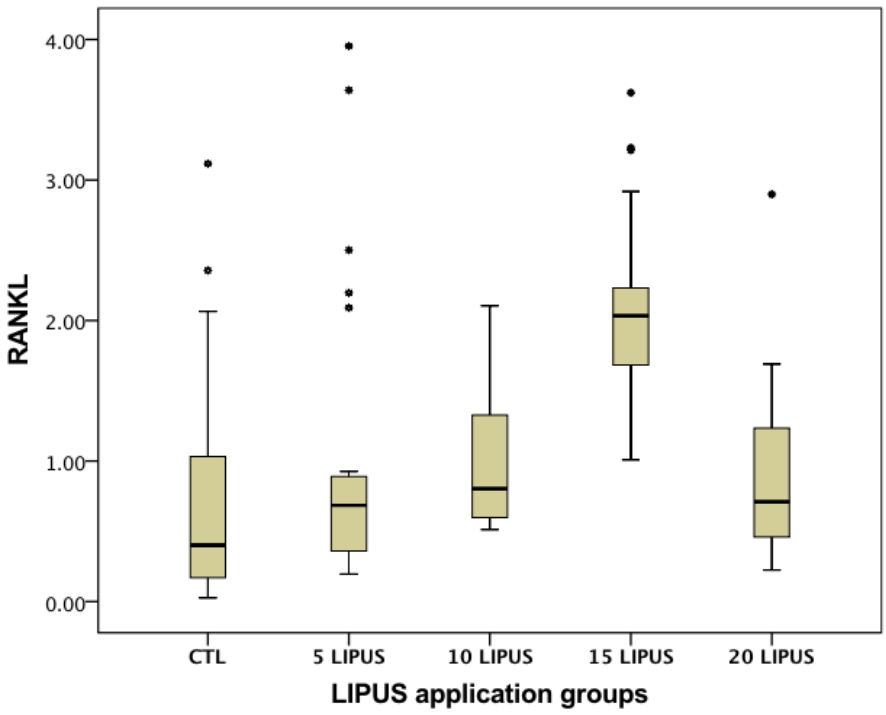

Figure 4.6: Box plot of the expression results of RANKL among the five groups showing very small expression of this gene. (CTL: Control, # LIPUS: # minutes of LIPUS application)

Table 4.3: Statistical analysis results (Tamahane's test) of multiple comparison of the RT-PCR results of the significantly expressed genes DMP1, DSPP and Col 1 (Diff: mean difference, SE: standard error, P: P-value, Col 1: collagen type I. DMP1: dentin matrix protein. DSPP: dentin sialophosphoprotein.)

		CTL			LIPUS 5			LIPUS 10			LIPUS 15			LIPUS 20		
		Dif	SE	P	Dif	SE	P	Dif	SE	P	Dif	SE	P	Dif	SE	P
CTL	DMP1				1.62	4.09	1.0	-57.11*	4.64	<0.01*	3.32	4.18	0.996	-1.29	4.54	1.0
	DSPP				0.15	0.39	1.0	-0.519	0.42	0.92	-0.17	0.47	1.0	1.6*	0.38	<0.01*
	Col I				-52.98*	7.15	<0.01*	-39.2*	6.24	<0.01*	-11.58	5.64	0.374	7.8	5.77	0.867
LIPUS 5	DMP1	-1.62	4.09	1.0				-58.74*	4.38	<0.01*	1.69	3.89	1.0	-2.92	4.27	0.999
	DSPP	-0.15	0.39	1.0				-0.67	0.39	0.614	-0.33	0.44	.998	1.45*	0.34	<0.01*
	Col I	52.98*	7.15	<0.01*				13.78	6.54	0.334	41.4*	5.97	<0.01*	60.79*	6.09	<0.01*
LIPUS 10	DMP1	57.11*	4.64	<0.01*	58.74*	4.38	<0.01*				60.44*	4.47	<0.01*	55.82*	4.8	<0.01*
	DSPP	0.51	0.42	0.92	0.67	0.39	0.614				0.34	0.46	0.998	2.12*	0.37	<0.01*
	Col I	39.2*	6.24	<0.01*	-13.78	6.54	0.334				27.61*	4.84	<0.01*	47*	5	<0.01*
LIPUS 15	DMP1	-3.32	4.18	0.996	-1.69	3.89	1.0	-60.44*	4.47	<0.01*				-4.62	4.36	.969
	DSPP	0.17	0.47	1.0	0.33	0.44	.998	-0.34	0.46	0.998				1.78*	.43	<0.01*
	Col I	11.58	5.64	0.374	-41.4*	5.97	<0.01*	-27.61*	4.84	<0.01*				19.39*	4.23	<0.01*
LIPUS 20	DMP1	1.29	4.54	1.0	2.92	4.27	0.999	-55.82*	4.8	<0.01*	4.62	4.36	.969			
	DSPP	-1.6*	0.38	<0.01*	-1.45*	0.34	<0.01*	-2.12*	0.374	<0.01*	-1.78*	0.43	<0.01*			
	Col I	-7.8	5.77	0.867	-60.79*	6.09	<0.01*	-47*	5	<0.01*	-19.39*	4.23	<0.01*			

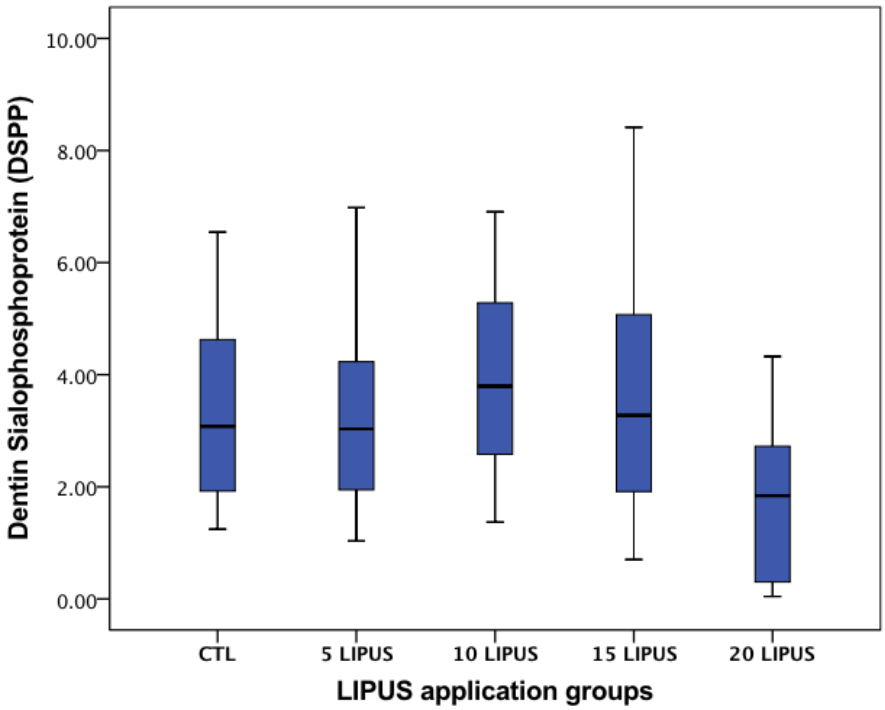

Figure 4.7: Box plot of the expression results of DSPP among the five groups showing very small expression of this gene and 20 LIPUS being the lowest. (CTL: Control, # LIPUS: # minutes of LIPUS application)

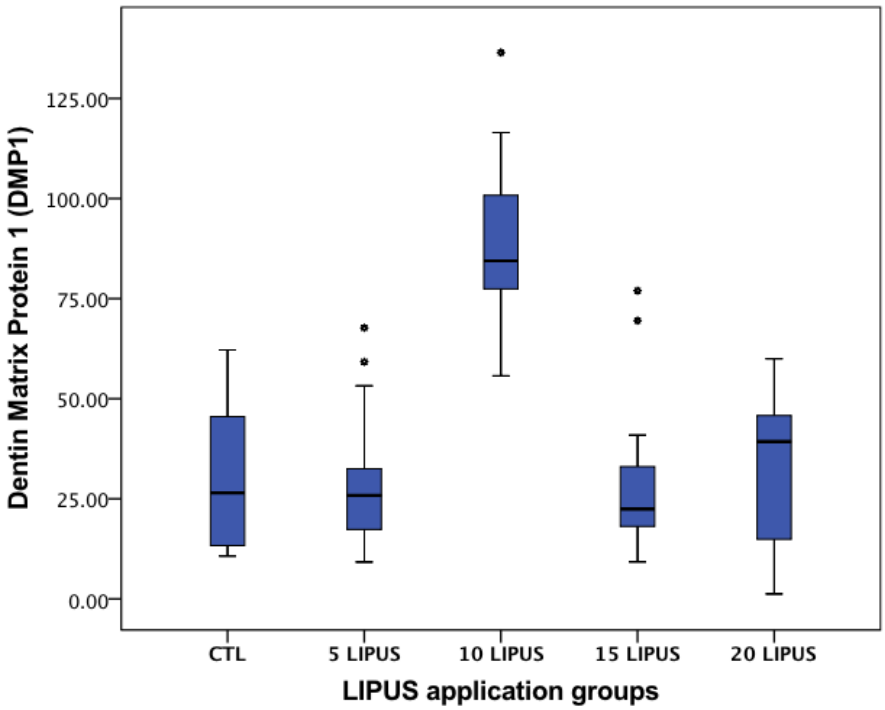

Figure 4.8: Box plot of the expression results of DMP1 among the five groups showing very small expression of this gene. (CTL: Control, # LIPUS: # minutes of LIPUS application)

Finally, Collagen type I was expressed in all groups and it was detected in greater amounts than the other genes of interest. The 5- and 10-minute LIPUS application groups were the highest and they had almost two-folds the expression of the rest ($P < 0.01$), also the 5 LIPUS group was higher than the 10 LIPUS group but the difference was not statistically significant ($P = 0.334$). The 15-minute LIPUS application group had a higher expression than the control and the 20-minute LIPUS application groups. However, the difference was not statistically significant compared to the control group ($P = 0.374$) but it was from the 20-minute LIPUS group ($P < 0.01$). Lastly, the control group had a higher expression than the 20-minute LIPUS group but the difference was not statistically significant ($P = 0.867$). (Figure 9, Table 3)

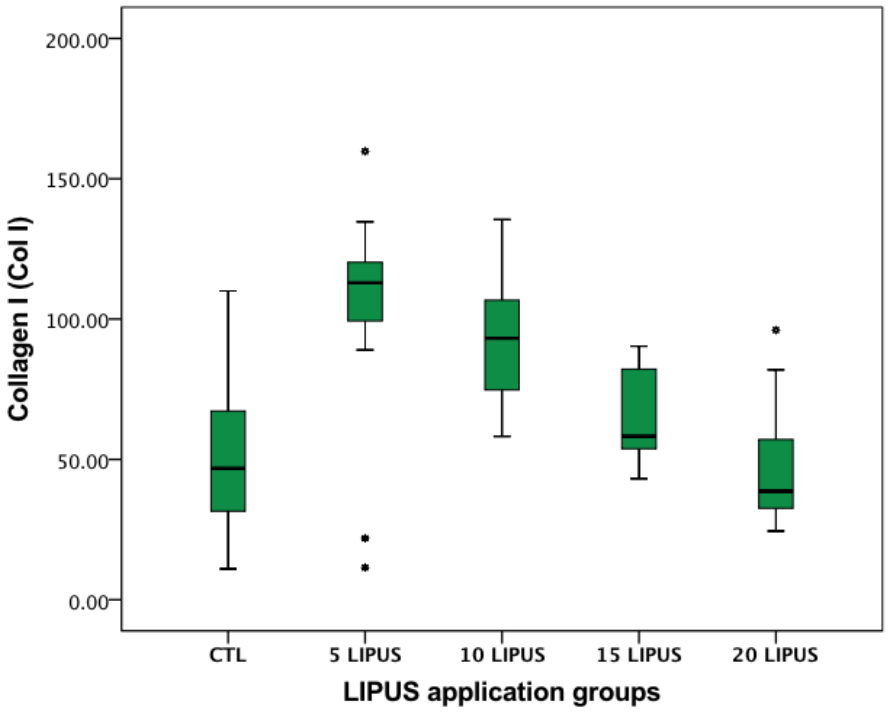

Figure 4.9: Box plot of the expression results of Collagen I among the five groups showing higher expression in the 5 and 10 LIPUS groups with few outliers overall. (CTL: Control, # LIPUS: # minutes of LIPUS application)

4.4 Discussion:

When acoustic waves pass through the tissue, energy absorption by the different tissue layers is proportional to the density. This differential rate of absorption may play a critical role in targeting the cells inside and around the hard tissue of the dentoalveolar complex. Although the actual mechanism of LIPUS effect on tissue is not known, one of the documented mechanisms of stimulation is thermal effect. One of the previously studied mechanisms was the thermal effect, but thermal effects were reported to occur when using intensities as high as 1–3W/cm^2, which can cause considerable heating of tissues. In this study we used only 30 mW/cm^2 and likewise in other studies using LIPUS (20–50mW/cm^2) heating effect did not reach 1˚C.[13] Likewise in our study the average increase of the media temperature was 0.41˚C ± 0.36. This study tested the hypothesis of whether LIPUS has a stimulatory effect on the dentin-pulp complex using a 3-D *in vitro* model after a single application. The concept of stimulating the secretion of extracellular matrix by dental forming tissues will result in a reparative potential of the hard dental tissue, which is not comparable to highly remodelling hard tissues like bone.

We investigated the effects of LIPUS on the dentin-pulp complex in this *in-vitro* model and measured its impact on odontoblast cell number and secretory activity manifested by the predentin layer thickness. Histomorphometric analysis revealed non-significant changes due to LIPUS application in the two measured variables; odontoblasts cell count and predentin thickness. Although the histological pattern and architecture were somehow different than the traditional

normal pulp tissue layout, it was not different among the five groups. Although stimulatory effects were found due to ultrasound application on odontoblast-like cells after causing initial 3-4 % cell death [14,15], this experiment is different in three ways. Firstly, the tissue tested in our study is a 3-D human tissue *in vitro* model and not the more viable rats odontoblast cell lines whereas previous research reported differences in response when comparing cell line and 3-D tissue culture models.[16] Secondly, the characteristics and technique of ultrasound application are different in our study compared to others. Finally, this study was for a short period of time, which was too short for producing structural changes but adequate for detecting gene expression changes compared to a similar long-term study we did.[17] Furthermore, studies that documented adverse effects histologically used higher intensities (0.2-2 W/cm^2) [18]; but in our study the LIPUS power emitted from the transducer did not exceed 33 mW/cm^2. Although there was no evidence of change in hard tissue layout or histological pattern indicative of LIPUS stimulation, gene expression results confirmed a stimulatory effect of LIPUS on the dentin-pulp complex *in vitro*. The RT-PCR results showed that mRNA expression of one of the major structural proteins of dentin; collagen type I was expressed more in the 5- and 10-minute LIPUS application groups. Higher intensities of LIPUS stimulated type I collagen mRNA expression in cementoblasts *in vitro* [19], odontoblasts (the output power was not specified in this study) [15] but not in human gingival fibroblasts [20].

The major dentin matrix non-collagenous structural proteins that we investigated their mRNA expression like DMP1 and DSPP, has a regulatory effect

on development and differentiation of the odontoblasts [21], the initiation of hydroxyapatite nucleation [22] and regulates the expression of osteocalcin, alkaline phosphatase ALP [23] and interacts specifically with collagen and initiates hydroxyapatite crystals and controls the rate of crystal growth [24]. From the RT-PCR results in our study, mRNA for DSPP was expressed in very low amounts in all groups with the 20-minute LIPUS application being the lowest among them. On the other hand mRNA for DMP1 was expressed in larger amounts and was significantly higher in the 10-minute LIPUS application group. This is in agreement with previous study that showed stimulatory effects of 5- and 10-minute LIPUS application on the expression of ALP and osteopontin OPN by human gingival fibroblasts [20].

Transforming growth factor -β1 was not expressed in any of the five groups, although reports indicate that low frequency ultrasound (30 kHz) increases the expression of TGF β1 by odontoblast in a cultured cell line. [15] TGF-β1 was of interest in this study because it has been shown to influence the behaviour of dental pulp cells during dental pulp repair including.[25, Nie, 2006 #1602,26] Also, TGF-β1 was found to stimulate matrix secretion and initiate odontoblast cytodifferentiation *in vitro* and *in vivo.*[7]

Tumor necrosis factor receptors-ligand family expression showed that the 15-minute LIPUS application group had higher expression amounts of mRNA of OPG and RANKL. Although the difference was significant we believe this is not conclusive for any inhibitory or stimulatory effect of LIPUS. The balance of RANKL/OPG can't be assumed different because of the very low expression of

RANKL in all groups except the 15-minute LIPUS, which was also the only group that expressed OPG in very low amount. Low intensity ultrasound at 1.5 MHz/30 mW/cm^2 was found to increase the expression of RANKL and decrease OPG expression by SaOS-2.[27] On the other hand, higher power of ultrasound at 62.5 or 125 mW/cm^2 markedly inhibited RANKL plus M-CSF-induced osteoclastic differentiation from bone marrow stromal cells.[28] Moreove, LIPUS has been found to alter the OPG/RANKL ratio enhancing reparative process of the orthodontically induced root resorption in rats.[29] The results of our study would be suggestive of a very low odontoclastogenesis potential of the permanent teeth dentin-pulp complex.

4.5 Conclusion:

In summary, tooth slice organ culture (TSOC) is an easy and reproducible yet technique sensitive *in-vitro* model that can be used to investigate the dentin-pulp complex while maintaining the relationship between the odontoblast and the dentin matrix which is crucial in maintaining the normal function of odontoblasts. Human TSOC differs from the rat TSOC model where the rat incisor has the ability to grow through the animal's life, but the human TSOC is a more realistic representation of the human dentin-pulp complex with limited regeneration abilities. We found that short term results of one application of LIPUS for 10 minutes increases the expression of Collagen I and DMP1. Also the 5-minute LIPUS application group increased the expression of Collagen I. DSPP were expressed in low amounts but was the lowest in the 20-minute LIPUS application

group. Also the low expression of RANKL and OPG where expression was highest in the 15-minute LIPUS application group, but the expression in all the groups was very low which is indicative of the low odontoclastogenesis potential of the dentin pulp complex.

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Chapter 5:

Effect of Low Intensity Pulsed Ultrasound on orthodontically induced root resorption in Beagle dogs

Part I: μCT volumetric analysis

5.1 Introduction:

Orthodontically induced root resorption is the second most common side effect of orthodontic treatment[1,2] after white spot lesions. The underlying mechanism behind this process is not fully known at the molecular level but it has been attributed mainly to the inflammatory process in response to hyalinized and compressed areas of the periodontal ligament (PDL) hence the name orthodontically induced inflammatory root resorption (OIIRR).[3] Individual variability in severity and progression of OIIRR at the clinical level guided researchers to investigate predisposing factors like genetics, anatomy and orthodontic mechanics.[4] Although severe forms of OIIRR are not very common[1,5] still they form a significant portion of the medico legal issues orthodontists are subjected to.[6,7]

Research investigated the relevance of clinical mechanics, genetics, anatomical variations, medication and medical and periodontal health status as predisposing factors that may serve as a predictor of severe root resorption cases.[4] Another pathway of research was focusing on the prevention, management and treatment of OIIRR. Most of the proposed or tested methods were invasive[8,9] or not clinically applicable[10,11] except for Low Intensity Pulsed Ultrasound (LIPUS).[12] LIPUS is a form of mechanical energy that is transmitted through and into living tissue as acoustic pressure waves where this energy is absorbed at a rate proportional to the density of the tissues in which it passes through. The micromechanical strains produced by these pressure waves in biological tissues were assumed to initiate biochemical events that affect hard and soft tissue

activity. This form of therapeutic ultrasound has already been very well established at the enhancement of bone fracture healing.[13]

Effects of LIPUS were extended from the effects on bone forming tissue and cells to dental forming tissues. LIPUS had an anabolic effect on human PDL cells, which provides important insight into the promotion of early cementoblastic differentiation of immature cementoblasts. [14] Moreover it had a stimulatory effect on cementoblasts themselves by inducing the production of alkaline phosphatase (ALP) which plays a role in hard tissue mineralization.[15] Although these studies were testing LIPUS effects *in vitro*, it was found *in vivo* that LIPUS stimulates angiogenesis during wound healing,[16] and it has an anti-inflammatory action.[17-19] A human[12] and animal[20] trials presented the preventive effect of LIPUS against OIIRR.

In this experiment we investigated the effect of LIPUS on orthodontically induced root resorption and the tooth movement *in vivo*. The study was designed to test two hypotheses; the first was if LIPUS reduces or prevents orthodontically induced inflammatory root resorption. The second one was if LIPUS has any effect on the rate of orthodontic tooth movement.

5.2 Materials and Methods:

5.2.1 Sample:

The study sample consisted of 10 adult male beagle dogs with an average age upon the start of the study 1Y-7M ± 8 days. The animal research ethics committee at the University of Alberta approved the study design and protocol. The animals were ordered from Marshall BioResources, North America and upon

arrival they were kept for two weeks prior to any procedures for conditioning purposes.

***5.2.2 Anesthesia and tooth preparation**:*

The animals were subjected to general anesthesia to prepare the teeth for full crown coverage. The dogs were premedicated with a sedative and antiemetic (Acepromazie/SubQ/ 0.05 mg/kg), analgesic (Hydromorphone/Sub Q/ 0.1 mg/kg) and muscarinic anticholinergic drug (Glycopyrrolate/Sub Q/0.01 mg/kg). The animals were intubated for the administration of the inhalation anesthesia Isoflurane (Benson Medical Industries Inc., Canada). The dogs were maintained on 1.5 litres of medical Oxygen and 1% to 3% Isoflurane as needed throughout the surgery. Full crown preparations were performed on the mandibular permanent first molar and fourth premolar (Fig. 5.1A) using a high-speed handpiece operating on NSK MIO coreless micromotor system with a tapered diamond burr cooled with saline (Fig. 5.1B).

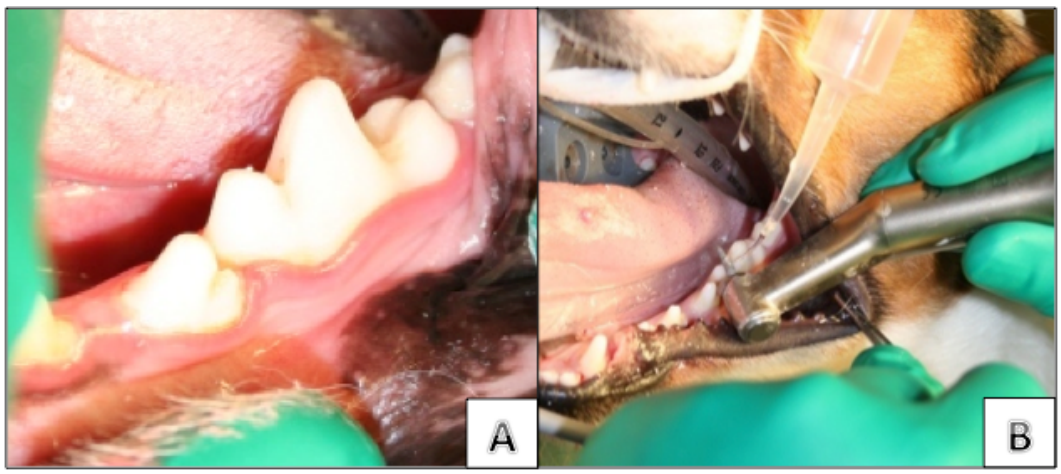

Figure 5.1: A: Full crown preparation on the mandibular 1st molar and 4th premolar. B: Crown preparation using tapered diamond stone on a high-speed handpiece with saline coolant.

After teeth preparation and impression procedures (Patterson Dental/Dentaire Canada Inc. Montreal, Canada) (Fig. 5.2) on each side, local anesthesia was administered to the mandibular third premolar using a dental syringe, short needle (22 mm) 30 gauge and anesthetic carpule Lignospan® standard, Lidocaine HCl and epinephrine injection (Lidocaine HCl 2% and epinephrine 1:100,000, cartridges 1.8 mL). The third premolars were sectioned into two halves separating the mesial and distal roots. Afterward the two segments were extracted using DNSPLY, Forceps No. 147; and, after removal of both segments, the extraction sockets were closed using resorbable 4-0 PDS suture material (Fig. 5.3). The oral cavity was cleared of the impression material and gauze then the inhalation anesthesia was discontinued and the animals were monitored until full recovery.

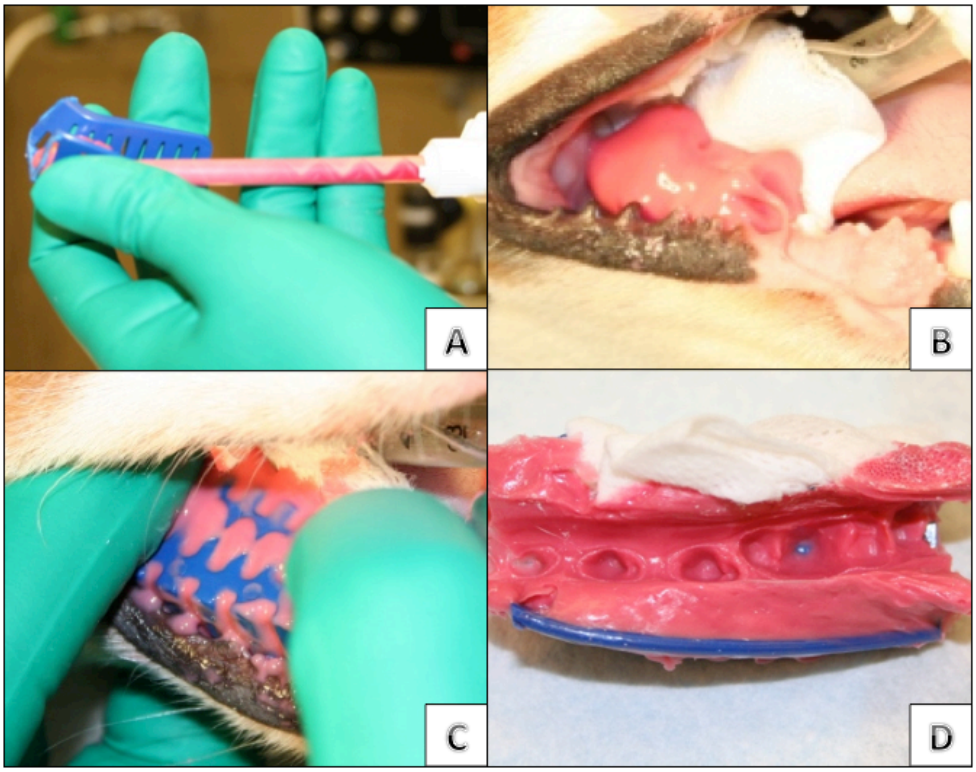

Figure 5.2: A: PVS impression material loaded in plastic tray after cleaning and drying out the prepared teeth. B: Teeth were covered with the impression before seating the tray. C: Tray fully seated with digital pressure and held in position for 5 minutes. D: Impressions were evaluated after setting for the presence of any defects.

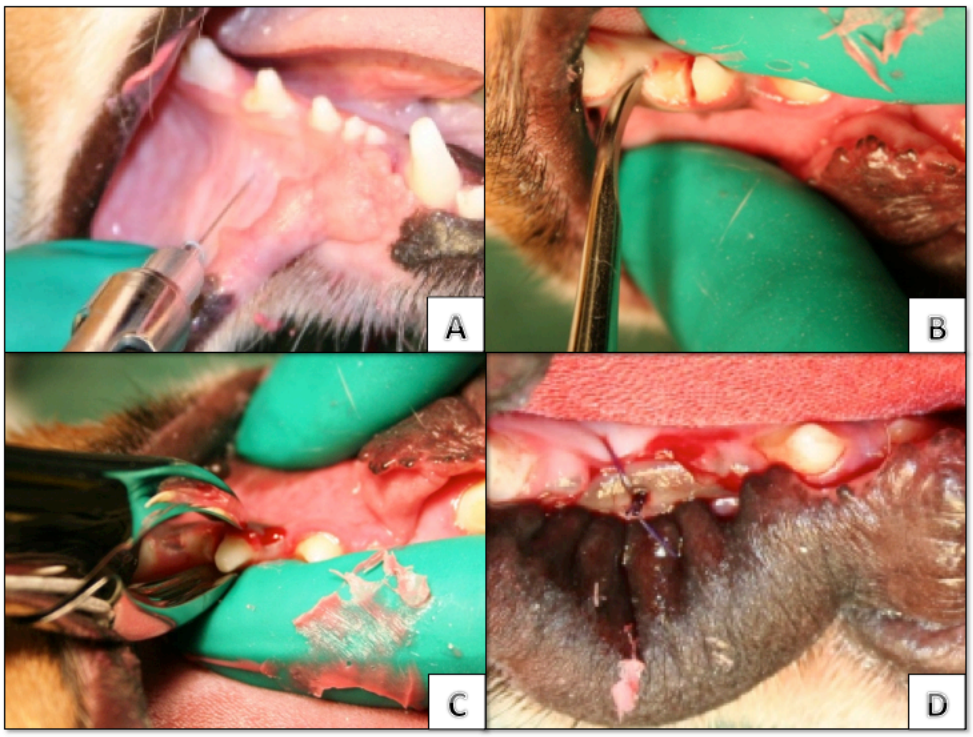

Figure 5.3: A: Local anesthesia infiltration using dental syringe with a short needle. B: Mandibular third premolar were sectioned in the furcation area and luxated using an elevator. C: Mesial and distal halves were extracted separately. D: Extraction socket were closed using 4-0 resorbable sutures.

5.2.3 Recovery and healing:

During recovery a postoperative analgesic (Metacam 0.1 mg/kg, orally) and antibiotics (Convenia 0.1 ml/kg, SubQ) were given to the animals. The animals were assessed twice on the day of the operation and daily for the next 4 days for clinical signs of pain and wound healing. The animals were maintained in normal conditions and a soft diet for a healing period of 54 days during which time the orthodontic appliances were fabricated.

5.2.4 Orthodontic appliance and force system:

Full crown wax ups were done for the first molar and the fourth premolar on the stone models poured out of the impressions. After that GAC Dentsply .022" x .028" bondable mandibular molar tubes were glued on the buccal surface of the full crown wax ups with a straight 0.021" x 0.025" stainless steel wire in the tube arch wire slot holding the two tubes in the same vertical and horizontal orientation to avoid any first-, second-, or third-order initial force on the fourth premolar. Afterward the crowns were casted into low fusing metal alloy. The crowns were finished and polished and the fitting surfaces were sandblasted with 50-µm aluminum oxide particles.

Under general anesthesia the prepared teeth were cleaned using pumice and rubber cups then rinsed with water then dried out to try in the crowns. We cemented them with dual cure permanent resin cement system (NX3 Nexus® Third Generation) and adhesive system (OptiBond All-In-One). The cement flash was removed using a plastic dental instrument and dental floss, then the

mesiodistal dimension of the two crowns on each side were measured using a digital caliper. Segment of a straight archwire 0.021" x 0.025" (3M Unitek, Canada) stainless steel wire was inserted in the attachment tube with an Ni-Ti open coil spring (Ormco, Canada) compressed between the two tubes to deliver a force of 0.98 Newton measured with force gauge (Fig. 5.4). The opposing teeth were reduced using the diamond burr to remove any occlusal interference with the crowns and clearing the fourth premolar of any interference during movement. The orthodontic tooth movement was continued for 4 weeks. Every week the coil springs were evaluated and re-adjusted by adding a stop distal to the open coil spring if needed to maintain a force level of around 1 Newton /side. Teeth movements were measured using a digital caliper that can read up to 0.01 mm; the movements were calculated from the difference between the maximum distance from the distal surface of the first molar to the mesial surface of the 4th premolar at the gingival margin.

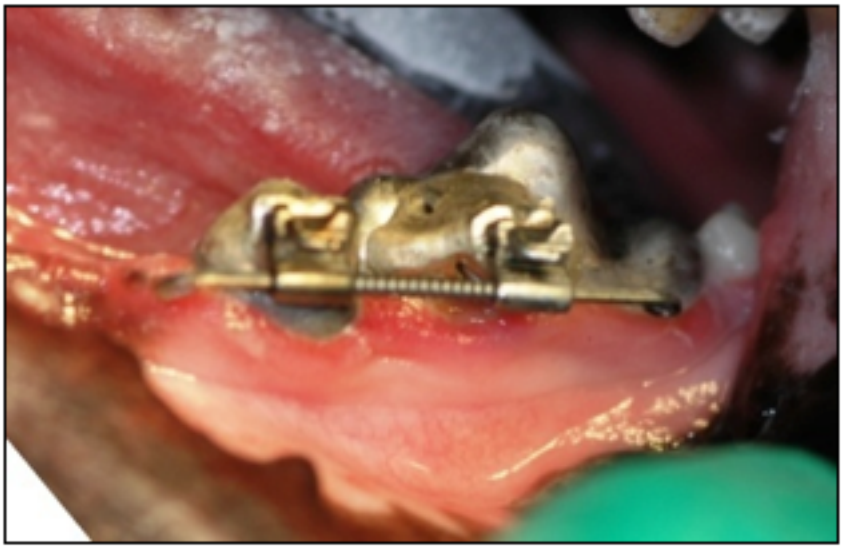

Figure 5.4: Cemented crowns on the 1st molar and 4th premolar with GAC tubes. Open-coil spring (Ni-Ti) on the 21x25 SS sectional wire.

5.2.5 LIPUS application:

The animals were sedated daily using DOMITOR® (medetomidine hydrochloride) (0.25 mg/kg IM) before the application of LIPUS. The test side was exposed to LIPUS daily for 20 minutes in a single application for the period of the study (4 weeks). We utilized a custom-built ultrasound device that provides adjustable output parameters and long-term operation stability (Smilesonica Inc., Edmonton, AB, Canada). The LIPUS device was set to generate ultrasound pulses with a repetition rate of 1 KHz. Each pulse had a square envelope with duration of 200 microseconds and a pulse frequency of 1.5 MHz. The ultrasound transducer had an emitting area of 1.56 cm^2 and it generated a temporal average ultrasound power of 47 mW (or a temporal average ultrasound intensity of 30 mW/cm^2). The transducer was applied intraorally on the buccal side of the fourth premolar with high viscosity gel (National Therapy Products Inc., Woodbridge, ON, Canada) as a coupling media (Fig. 5). The control side had a sham transducer applied to it for the same period of time. After the application of LIPUS, sedation was reversed using ANTISEDAN® (atipamezole hydrochloride) (1 mg/kg IM). Before, during and at the end of the experiment the LIPUS application device was inspected for consistency of electrical waveforms (1 KHz modulation, 200 microseconds pulse duration, and 1.5MHz carrier frequency) using a digital oscilloscope, and calibrated for ultrasound intensity of $30mW/cm^2$ using an ultrasound power-meter (model UPM-DT-1AV from Ohmic Instruments, Easton, MD, USA). The inspections and calibrations on a weekly basis confirmed that the ultrasound device provided stable ultrasound power output and maintained the desired

electrical parameters during the experiments. Moreover, we monitored the LIPUS output through the lingual surface of the mandible during the application session (Figure 6). After 4 weeks, the animals were euthanized by injecting 2-3 ml/4.5 kg Euthanyl into the cephalic vein. Afterward the mandibles were dissected and sectioned, using a bone saw, into blocks containing the fourth premolars and the supporting alveolar bone. The samples were stored into freshly prepared 4% paraformaldehyde.

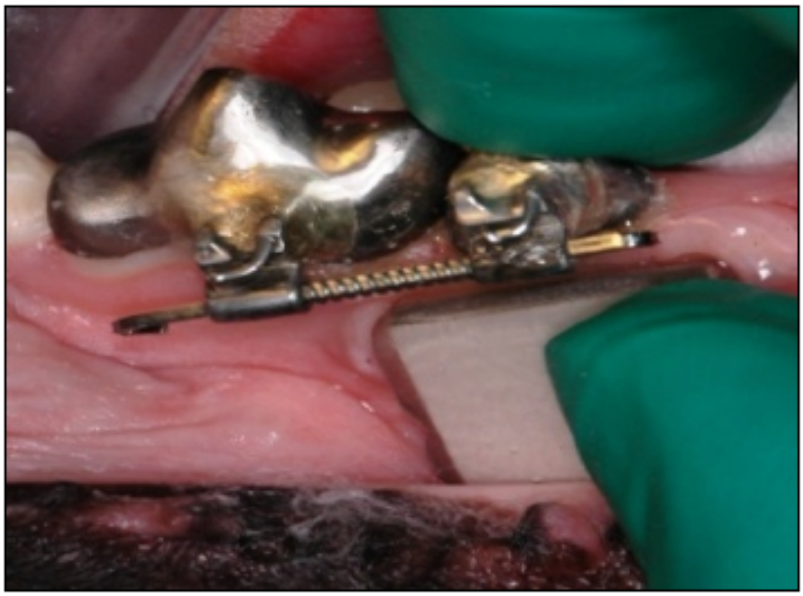

Figure 5.5: LIPUS applied from the buccal side of the 4th premolar daily for 20 minutes on the test side while the control had a sham transducer applied to it for the same period.

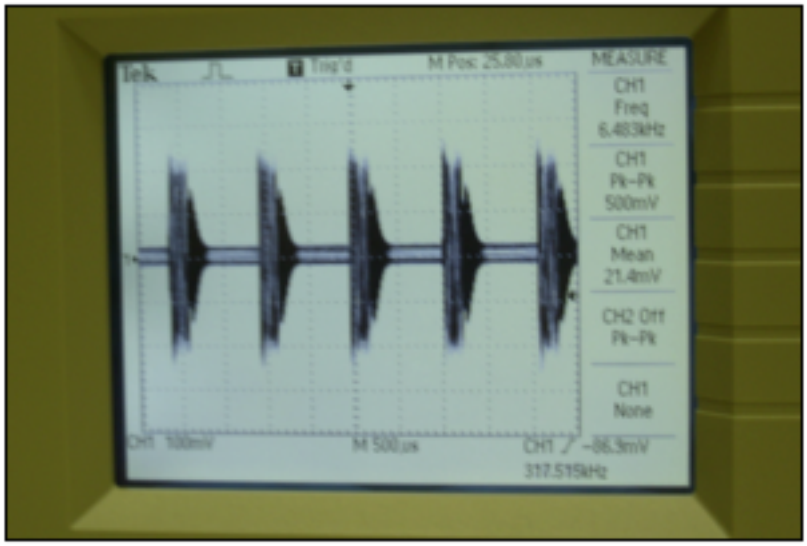

Figure 5.6: LIPUS output was monitored during the application by placing a sensor attached to a digital oscilloscope on the lingual side of the test side.

5.2.6 Micro–computed tomography:

Fixed samples were scanned using a high-resolution compact fan-beam tomogram (μCT, SkyScan 1072, Aartselaar, Belgium) and associated software (Version 2.6.0) at a resolution of 9μm using an x-ray source potential of 100kV, amperage of 100μA, and power of 10W through 180^{o} with a rotation step of 0.9^{o} at x12 magnification, to produce serial cross-sectional images composed of isotropic 19.4 mm^3 voxels. An aluminum filter of 1.0 mm thickness was used, and scans averaged 3 times. Scanned images were saved in *.tiff format. Scion Image, beta 4.0.2 (Scion Image Corporation, USA) were used to median-filter the raw image data to reduce noise. The filtered image data were rendered in three dimensions. Using this orientation, the 2D image stacks were exported to a commercial image analysis package (IP-PLUS, Media Cybernetics, Bethesda, MD, USA). Finally the images were reconstructed using NRecon© (Version 1.4.4) from SkyScan®. Reconstructed images were analyzed using CT Analyser (Version 1.6.1.0, Skyscan N.V. Kontich, BE). Resorption lacunae were analyzed by going through every slice over the whole length of the roots, starting at the slice where the root is covered with bone at all surfaces, and proceeding to the root apex. The RL was covered with ROI tool in all slices with the RL present coronally and apically, and then the algorithm in CT Analyser calculated the 3D soft tissue and hard tissue volumes within the ROI based on the radiographic density (Figure 5.7). From these images we calculated the number of resorption lacunae (RL), volume of RL and the percentage of root resorption to the total root volume. Also we measured the width of the PDL space on the mesial and distal

surfaces of the roots at five levels (Figure 5.8), the planes where we took the measurements started just below the alveolar crest and splitting the root in fifths according to the clinical root length (Figure 5.9).

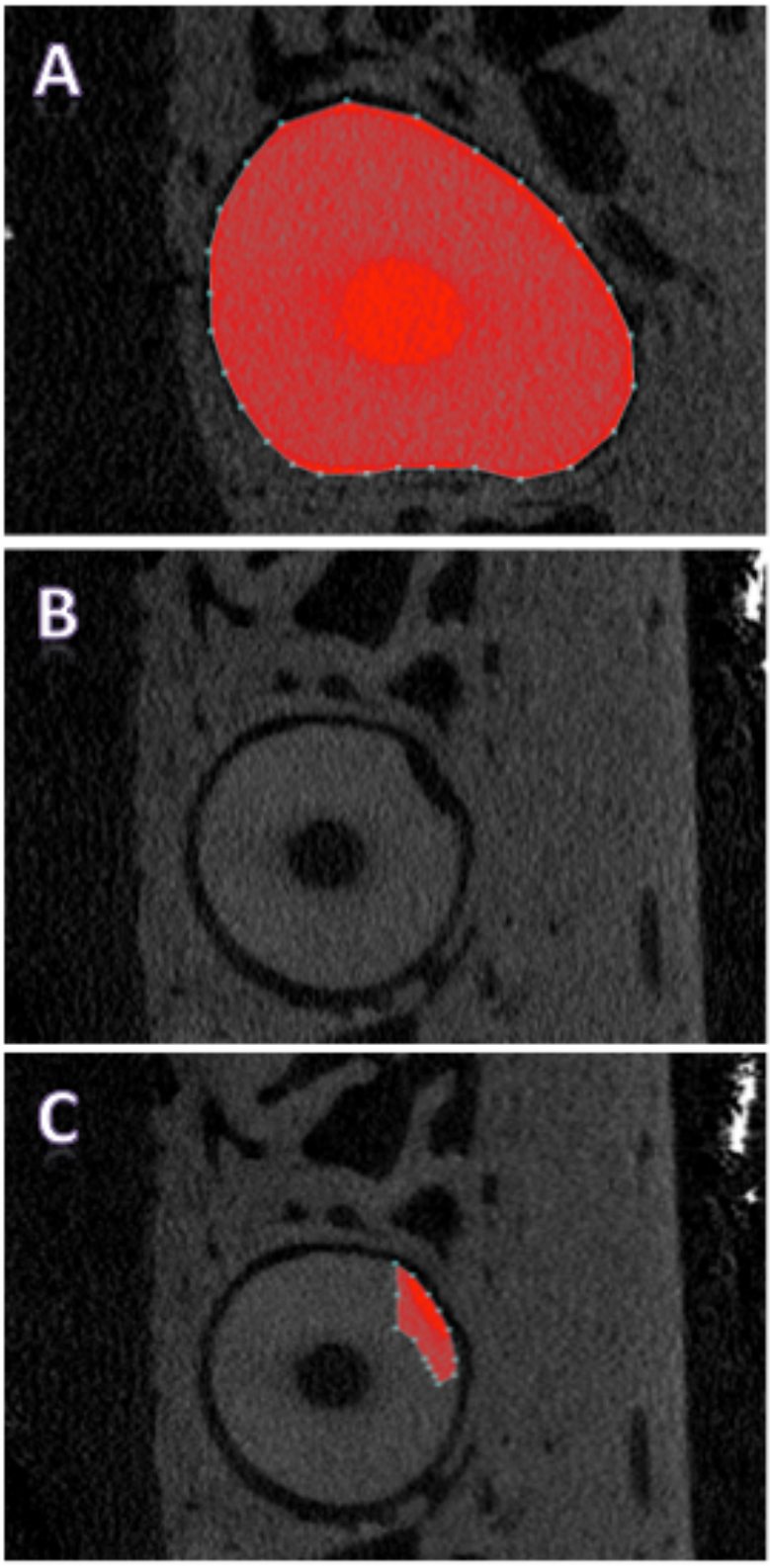

Figure 5.7: Cross sectional view of the micro-CT scan showing the process of volume measurement. A: The region of interest ROI in this figure is done while taking the whole root volume by covering the root in all slices from the furcation to the root apex. B, C: This picture shows how the RL was covered with ROI that was designed to restore the original contour of the root, then the algorithm in CT Analyser (Version 1.6.1.0, Skyscan N.V. Kontich, BE) will calculate the 3D soft tissue and hard tissue volumes within the ROI.

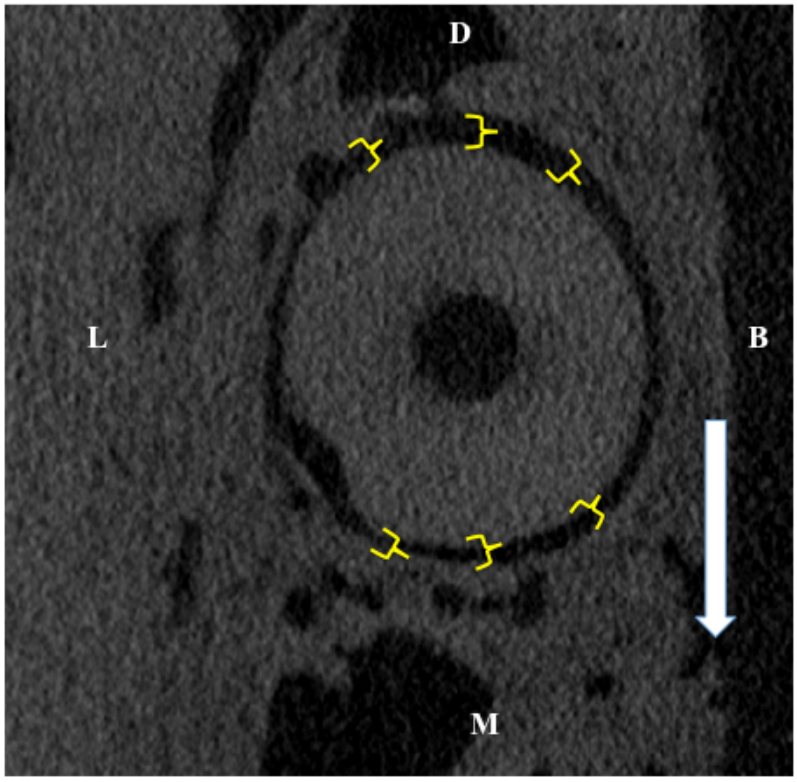

Figure 5.8: Cross sectional view of the micro-CT scan showing the areas of PDL space measurements on the mesial and distal root surfaces. The measurements were taken at three areas and on each area we took an average of three readings. Note that all the selected areas we avoided resorption lacunae on the root surface and the adjacent bone surface of the socket to minimize outliers and measurement errors. (*Arrow:* The force vector, M: Mesial, D: Distal, B: Buccal, L: Lingual)

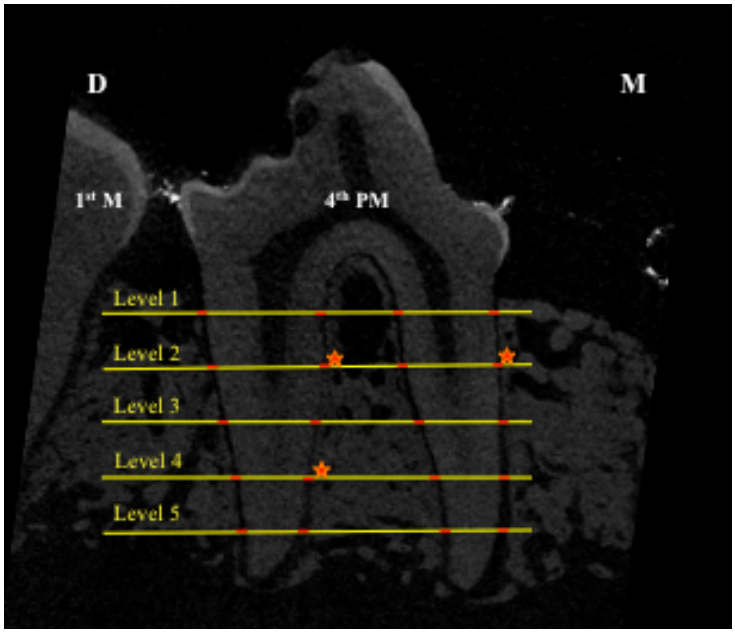

Figure 5.9: Sagittal view of the fourth premolar showing the levels at which the PDL space measurements were taken. The areas marked by (★) are the areas of PDL where the control group had statistically significant wider PDL spaces than the LIPUS group.

5.2.7 Blinding and intra-rater reliability measurements:

Each animal's right and left sides were allocated randomly to either the treatment (LIPUS) or control (no LIPUS). Tooth movement measurements, PDL width and resorption variables from the micro-CT were measured by the same investigator three times with a 2-week interval between each measurement. Measurements were taken on coded samples to test the intra-rater reliability and measurement error. Another investigator coded the samples before starting data collection for evaluator blinding.

5.2.8 Statistical Analysis:

Collected data were processed using SPSS 18.0, intra-rater reliability for all the variables were measured using intraclass correlation coefficient (ICC). Due to the small sample size and the split mouth design of the study we used non-parametric related samples test (Wilcoxon Signed Ranks) to analyze the collected data.

5.3 Results:

5.3.1 Reliability and error:

Intra-rater reliability test results varied among the measured variables where there was an absolute agreement in measuring the number of resorption lacunae on each root. On the other hand, it was the lowest in measuring the orthodontic tooth movement (ICC = 0.984). Measurement reproducibility of the other variables were fairly high where the ICC was ranging from 0.96 to 0.998 in measuring the PDL space and were 0.998 in measuring the RL volume and its

percentage to the total root volume (Total root volume = the residual root volume + total volume of resorption lacune).

5.3.2 Orthodontic tooth movement:

The teeth with LIPUS application moved more than the control with a mean difference of (0.188 ± 0.225 mm) but this difference did not reach statistical significance (P = 0.05) (Figure 5.10A, Table 5.1).

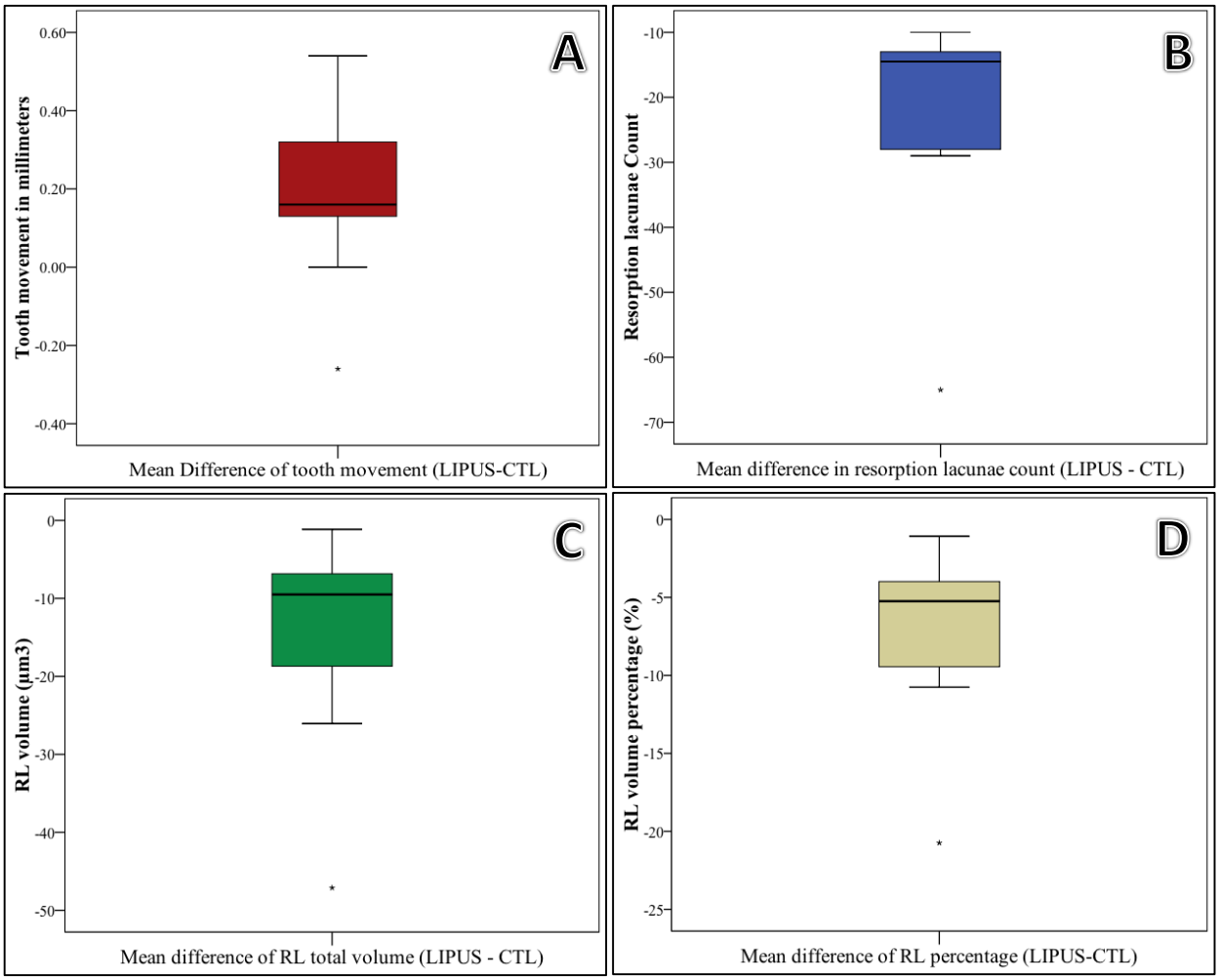

Figure 5.10: Box plot of the difference between LIPUS side and control side (CTL) {The difference variables were calculated as LIPUS – CTL} of the measured clinical and micro-CT variables: A: Orthodontic tooth movement. B: Number of resorption lacunae. C: Total volume of resorption in μm^3. D: Percentage of root resorption to the total root volume.

5.3.3 Root resorption:

Root resorption variables indicated that the LIPUS group had less root resorption compared to the control group. The RL count on the roots of the fourth premolar was less on the LIPUS side (Figure 5.10B, Table 5.1) by a mean difference of (22.7 ± 16.5 RL). Also the RL total volume was less in the LIPUS group too (Figure 5.10C, Table 1) by a mean difference of (14.4 ± 13.5 μm^3), the percentage of the resorption to the measures root volume per tooth (Figure 5.10D, Table 5.1) was also less in the LIPUS group by a mean difference of (7.4 ± 5.6 %). All of these root resorption variable differences were statistically significant (P = 0.005).

Table 5.1: Non-parametric statistical analysis results (Wilcoxon Signed Ranks Test) of the tooth movement and resorption measured variables (RL: resorption lacunae, SD: standard deviation) {The difference variables were calculated as LIPUS – CTL}.

	LIPUS		Control		Wilcoxon Signed Ranks Test
	Mean	SD	Mean	SD	P-value
Tooth Movement (mm)	0.79	0.17	0.6	0.21	0.05
RL count	9	4.44	31.7	14.8	<0.01*
RL total volume (μm^3)	5.44	2.94	19.8	13.1	<0.01*
RL percentage (%)	3.26	1.77	10.63	5.1	<0.01*

Table 5.2: Raw data for the 10 animals of the tooth movement and resorption measured variables (RL: resorption lacunae, SD: standard deviation).

	Movement (mm)		**RL #**		**RL volume (µm3)**		**RL %**	
	LIPUS	**Control**	**LIPUS**	**Control**	**LIPUS**	**Control**	**LIPUS**	**Control**
1	.54	.40	8.00	36.00	6.81	12.67	4.86	8.84
2	1.12	.58	8.00	23.00	2.23	14.02	1.81	10.82
3	.63	.50	7.00	17.00	2.25	12.00	1.30	7.19
4	.80	.48	9.00	22.00	4.56	11.56	1.88	5.87
5	.70	.46	6.00	19.00	4.49	5.64	2.98	4.06
6	.74	.56	3.00	68.00	1.77	48.88	1.04	21.78
7	.65	.51	20.00	33.00	8.85	15.69	6.09	10.30
8	1.00	.55	11.00	25.00	8.85	18.12	4.88	9.48
9	.90	.90	10.00	37.00	9.57	35.62	4.79	15.54
10	.81	1.07	8.00	37.00	5.11	23.80	2.99	12.44

In regard to the distribution of the root resorption lacunae, the pattern was different between the LIPUS and the control group. The RL count was the highest on the buccal surface in both groups. The mesial surface had higher RL compared to the lingual and distal surfaces in the control group. The mesial and buccal surfaces each had an average of 30% of the total RL where the rest was distributed on the lingual (21%) and distal (19%) surfaces. The RL distribution patterns were different in the LIPUS groups given that the overall RL count was significantly lower, the mesial and distal surfaces each had the lowest average RL count (16%) but the buccal surface had the highest (43 %) and the rest was distributed on the lingual surface (25%) (Figure 5.11, Table 5.3). Furthermore, we found that the RL distribution data of the animals # 6 and # 10 were outliers compared to the rest of the sample (Table 5.3). By omitting the data of these two samples, the trend of the RL distribution being highest in the buccal and mesial surfaces will change and the RL count on all four surfaces will not show any dominance.

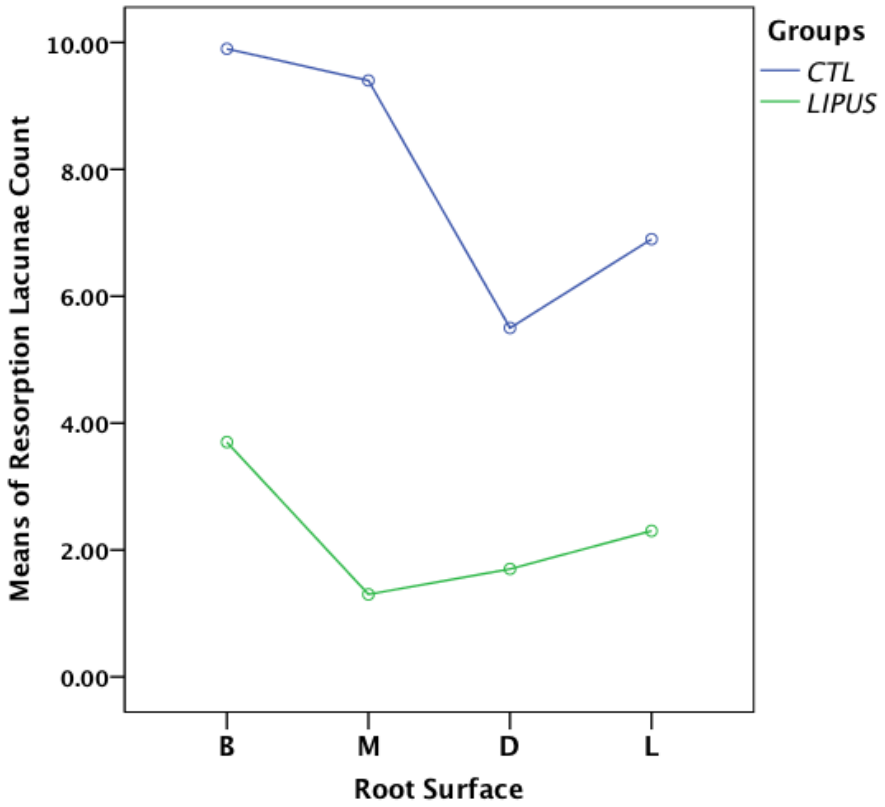

Figure 5.11: Profile plot showing the variation in the pattern of distribution of RL on the different root surfaces between the LIPUS and the control groups. RL on the mesial surface were the lowest in the LIPUS side where it's the second highest on the control group.

Table 5.3: The values of RL count on each root surface of the ten animals both control (CTL) and test (LIPUS) sides. (B: Buccal, M: Mesial, D: Distal and L: Lingual)

	Animal 1		**Animal 2**		**Animal 3**		**Animal 4**		**Animal 5**		**Animal 6**		**Animal 7**		**Animal 8**		**Animal 9**		**Animal 10**	
	LIPUS	**CTL**	**LIPUS**	**CTL**	**LIPUS**	**CTL**	**LIPUS**	**CTL**	**LIPUS**	**CTL**	**LIPUS**	**CTL**	**LIPUS**	**CTL**	**LIPUS**	**CTL**	**LIPUS**	**CTL**	**LIPUS**	**CTL**
B	3	7	3	9	4	5	6	2	4	2	4	22	3	9	1	8	4	12	5	23
M	0	11	1	8	2	8	2	13	1	4	0	24	6	3	1	8	0	12	0	3
D	0	8	1	3	0	0	5	6	1	8	4	4	0	6	0	4	4	12	2	4
L	5	10	2	3	0	4	7	1	4	5	0	18	0	15	1	5	3	1	1	7

5.3.4 PDL space:

Radiographic measurements of the PDL space in the μCT images showed that there were no significant differences between the LIPUS and the control teeth in all the measured areas at the five different levels along the root surfaces (P > 0.05) except at three locations. The three locations were at the second level on the mesial surface of the mesial root (P = 0.002), the mesial surface of the distal root at the second and fourth levels (P = 0.037), which was wider in the control group by (0.06 mm, 0.04 mm and 0.03 mm) respectively (Figure 5.9 and 5-12, Table 5.4).

Table 5.4: Non-parametric statistical analysis results (Wilcoxon Signed Ranks Test) of the PDL space measurements on the mesial (M PDL) and distal (D PDL) surfaces of both roots at the different 5 levels (Level 1 coronal and Level 5 apical)[P: P-value, Dif: Mean difference LIPUS side – Control side].

		Level 1		**Level 2**		**Level 3**		**Level 4**		**Level 5**	
		Dif	**P**	**Dif**	**P**	**Dif**	**P**	**Dif**	**P**	**Dif**	**P**
Mesial Root	**M PDL**	-0.026	0.24	-0.057	<0.01*	-0.018	0.09	-0.017	0.17	0.008	0.64
	D PDL	0.027	0.24	-0.002	0.95	-0.0001	0.8	-0.004	0.72	-0.011	0.51
Distal Root	**M PDL**	0.00027	0.64	-0.041	0.037*	-0.022	0.14	-0.026	0.037*	-0.03	0.17
	D PDL	0.018	0.24	0.003	0.51	-0.005	0.64	-0.0017	0.87	-0.006	0.87

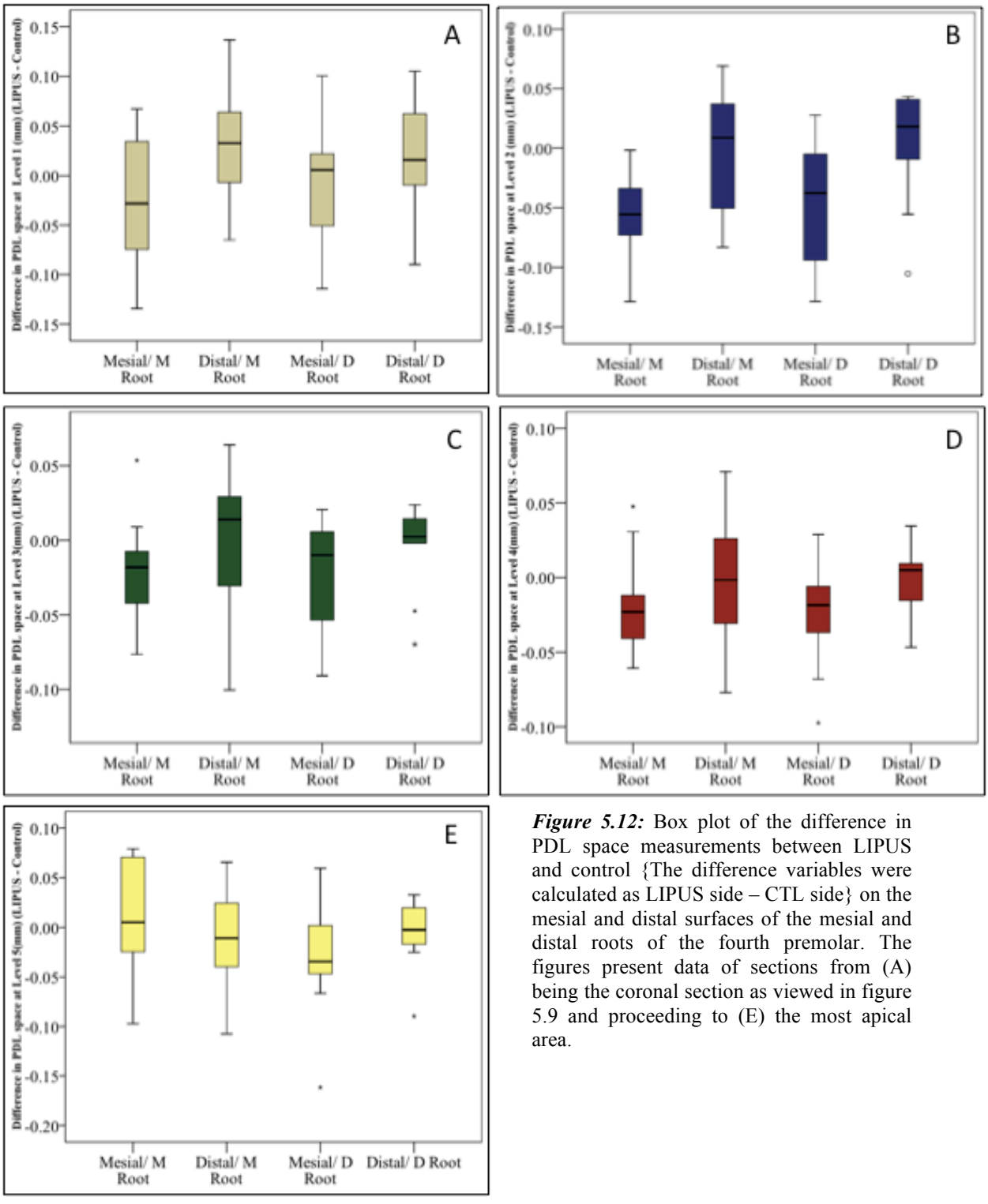

Figure 5.12: Box plot of the difference in PDL space measurements between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side} on the mesial and distal surfaces of the mesial and distal roots of the fourth premolar. The figures present data of sections from (A) being the coronal section as viewed in figure 5.9 and proceeding to (E) the most apical area.

5.4 Discussion:

The non-invasive nature and simple application of LIPUS makes it attractive to the clinician and patient. Previous investigations of LIPUS effects at the clinical level were done to evaluate the enhancement of bone healing after fractures, distraction or periodontal grafting.[21-24] Despite the multiple studies that demonstrated the anabolic and stimulatory effects of LIPUS, its actual intracellular mechanism is not known. Several theories were proposed for this mechanical energy effect at the molecular level.[13,25] In this model the biological system behind tooth movement and root resorption were studied as one system. This allowed the possible interaction between different tissues of the periodontium that may alternatively or simultaneously participate in the process of root resorption and repair.

The RL number variable represents the number of areas where the root resorption process was initiated but does not necessarily represent the severity of the process. Simultaneous application of LIPUS with continuous orthodontic force reduced the number of root resorption initiation areas. In previous histological studies, OIIRR has an inflammatory component as a response to the formation of hyalinized areas in the PDL.[26] The close relation of root resorption initiation sites and the areas of hyalinized PDL were documented histologically.[27] The LIPUS preventive role may be due to its anti-inflammatory characteristic,[17-19] angiogenic effect [16,28,29] or both.

The severity of root resorption was presented in micro-CT results by the total volume of the resorption lacunae and its percentage relative to the total

measured root volume. The extent of two processes, the root resorption process and the reparative process, will determine the final volume of root resorption. While maintaining continuous orthodontic force applied to the tested teeth, the reparative process can still exist at the same time in different areas or at the same resorption lesion.[30,31] This form of physiologic recovery and root repair would not occur as much as in a clinical situation when the tissue is given a chance while force decays. [26,30-32] LIPUS reduced the volume of resorption lacunae significantly. The possible mechanism could be either interfering with the resorption process, enhancing the reparative process or both. Several studies confirmed the anabolic effect of LIPUS on dental [29,33] and periodontal [14,15,34,35] cell lines *in vitro* and tissue *in vivo*.[12,21,36]

The direction of force applied and stress areas in the PDL determine the distribution pattern and severity of resorption lacunae.[37-39] Although OIIRR could occur in areas of tension and shear of the PDL, it is not as severe as in high stress and compression areas.[39] In this study the distribution was changed due to LIPUS application. Although the buccal surface has the highest number of RL in both control and LIPUS groups, the main compression site in the PDL (mesial) changed in the LIPUS from being the second highest to be the lowest in comparison to the control group. That can be explained by the fact that the fourth premolar is located buccal to the first molar which may result in pushing its root into the buccal plate of bone. Or due to possible rotation in the fourth premolar into a mesio-lingual direction and forcing the distal root into the buccal plate of bone. On the other hand, the raw data shows two outliers in RL distribution

(animals # 6 and # 10) which when omitted, the RL distribution trend changed significantly negating the dominance of the buccal and mesial surfaces. Although these two samples data shows as outliers from the rest, they still represent 20 % of the sample and omitting them can introduce some bias.

LIPUS output at the surface of the transducer was kept constant and calibrated throughout the study period, the actual intensity at the different tissue layers relative to root surfaces were unknown. Several studies on ultrasound propagation through materials were done using finite element analysis FEA models mainly. [40-42] Due to the heterogeneity of the dentoalveolar tissues considerable amount of error can occur as a result of its complexity. This error in calculating intensities at the different root surfaces may negate any significant differences.

Bone is a highly vascularized tissue and its remodelling lies behind the process of orthodontic tooth movement, although alveolar bone bending plays a role[43] a lot of credit is given to the PDL and its cell population.[44] Moreover the PDL response to tension and compression were found to be a major determinant of domination of osteoclastogenesis or osteogenesis during tooth movement. In this study, with highly reproducible measurements of the PDL space using the micro-CT, we tested the hypothesis of whether LIPUS can affect the pattern of frontal bone resorption and apposition at the PDL space under orthodontic force. The results showed no statistical significance except in two areas above the centre of resistance of the premolar on the compression side, where the PDL space was

wider in the control group. The results are not enough to make any assumptions about the pattern of bone resorption or apposition at the PDL interface.

In summary, this *in vivo* study demonstrated the preventive action of LIPUS against orthodontically induced root resorption Knowing that orthodontic treatment at clinical settings will take more than 12 months on average and our experiment was only for one month; the reduction in extent and severity of root resorption was significant without the contribution of physiologic recovery and root repair normally happening during orthodontic force decay and deactivation periods. This therapeutic effect may be extrapolated and become clinically significant on a long-term basis, but will need to be addressed with a long-term study. Also investigating in a long-term study can clearly define the effect of LIPUS on the rate of tooth movement, which can be another attractive clinical outcome in clinical orthodontics.

5.5 Conclusions:

Delivery of a single daily LIPUS application during continuous orthodontic force application provides a preventive effect against root resorption. Orthodontically induced root resorption was significantly reduced in severity and distribution. LIPUS is a non-invasive and clinically appropriate potential solution for treating orthodontically induced root resorption.

5.6 References:

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Chapter 6:

Effect of Low Intensity Pulsed Ultrasound on orthodontically induced root resorption in Beagle dogs

Part II: histology and immunohistochemistry

6.1 Introduction:

Orthodontically induced root resorption is the second most common side effect of orthodontic treatment [1,2]; and although severe forms are not very common [1,3] it forms a significant portion of the medico legal issues orthodontists are subjected to [4,5]. Unfortunately the underlying mechanism of this process is not fully known but it has been attributed mainly to the inflammatory process in response to hyalinized and necrotic tissue in the periodontal ligament (PDL) due to compression from orthodontic forces, hence the name orthodontically induced inflammatory root resorption (OIIRR) [6]. Individual variability in severity and progression of OIIRR at the clinical level was linked to some predisposing factors like genetics, anatomy and orthodontic mechanics [7].

From light and electron microscopic studies on the initial process of root resorption and removal of the hyalinized tissue, it has been found that during the remodelling process, root resorption may occur as a side effect of the cellular activity associated with removal of the necrotic tissue in the PDL. [8,9] The initial access of resorptive cells to the root surface occurs in the immediate periphery of the necrotic zone [10]. Root resorption beneath the main necrotic zone takes place at a later phase, during which multinucleated TRAP-positive cells remove the bulk of necrotic PDL tissue and resorbs the outer layer of adjacent root cementum [11]. It was found that TRAP enzyme is of great importance both for the removal of necrotic periodontal membrane tissue and of the surface part of root cementum [12]. During that process dentinal tubules could be exposed, a phenomenon being considered as an impetus for attracting progenitor cells for differentiation and

fusion into clast cells. Also the thinning of the cementum layer leaves a naked unprotected dentin [10,13] to which odontoclasts develop ruffled border structures. [14]. Observations based on cell cultures indicated that the clast cells that produced resorption lacunae were derived from mature monocytes and macrophages [15].

Low Intensity Pulsed Ultrasound (LIPUS) is an acoustic radiation with frequencies above the limit of human hearing that can transmit into the tissue as high frequency mechanical waves. While these mechanical waves are transmitted through tissue as acoustic pressure waves, its energy is absorbed at a rate proportional to the density of the tissues in which it passes through. The micromechanical strains produced by the pressure waves while passing through the biological tissues are assumed to initiate biochemical events that affect hard and soft tissue activity. This form of ultrasound has been already established as a therapeutic form of ultrasound that can enhance healing of bone fracture [16]. LIPUS were applied for dental purposes and were found to have an anabolic effect on human PDL cells *in vitro*, which provides important insight into the promotion of early cementoblastic differentiation of immature cementoblasts. [17] Moreover it had a stimulatory effect on cementoblasts themselves by inducing the production of alkaline phosphatase (ALP) that plays a role in hard tissue mineralization [18]. Also *in vivo* it was found that it stimulates angiogenesis during wound healing [19], anti-inflammatory action [20-22] reparative effect on the periodontium after flap surgery [23] and reparative effect on OIIRR [24].

In this experiment we investigated the effect of LIPUS on root resorption, periodontium and dental pulp of teeth subjected to bodily orthodontic movement

in vivo. The study was to test whether LIPUS has anabolic effects on cementum *in vivo*, prevents root resorption and facilitates its repair and stimulates the bone remodelling process around orthodontically moved teeth.

6.2 Materials and Methods:

6.2.1 Sample:

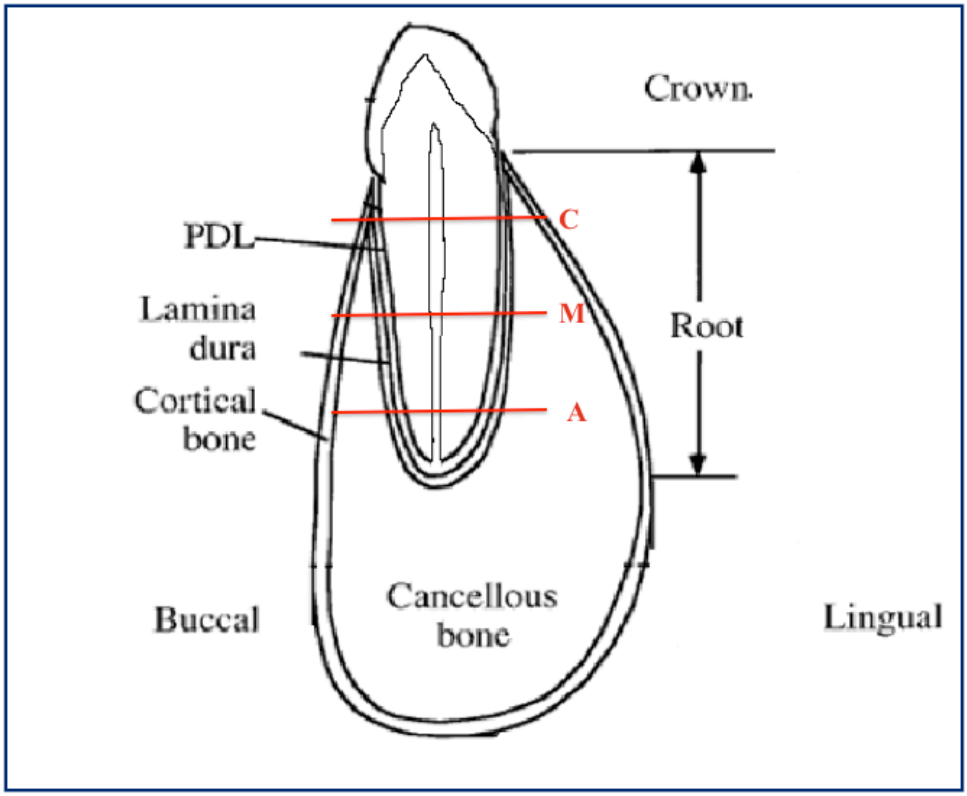
The study sample consisted of 10 adult male beagle dogs with an average age upon the start of the study of 1Y-7M ± 8 days. The animal research ethics committee at the University of Alberta approved the study design and protocol. The orthodontic and surgical procedures were performed as described in Chapter 5. After 4 weeks of daily application of LIPUS for 20 minutes to one side of each animal with the other side being the control, the animals were euthanized and the mandibles were dissected and sectioned, using a bone saw, into blocks containing the fourth premolars and the supporting alveolar bone. The samples were stored into freshly prepared 4% paraformaldehyde.

6.2.2 Histology and immunohistochemisty analysis:

Several slides were sectioned out of the tissue five-microns thick for Hematoxylin and Eosin (H & E) and immunohistochemical staining for tartrate-resistant acid phosphatase (TRAP). Tissue sections were performed at three levels of the root length and the levels were at the cervical (coronal) third, middle and apical thirds of the root. Slides were then de-waxed in xylene and hydrated to water through a series of alcohol gradients. Prior to staining, the tissue sections were pretreated in a Tris-EDTA pH 9.0 target retrieval solution (Dako K8004) for

2 hours at 70 °C. Once cooled to room temperature (RT), a 3.0% hydrogen peroxide solution was used on the sections for 5 minutes to quench any endogenous peroxidase activity. Serum Free Protein Block (Dako X0909) was used for 5 minutes at room temperature to neutralize any charged molecules on the tissue sections that may cause non-specific staining. Mouse anti TRAP antibody was added at a concentration of 0.32 mg/L. The isotype negative control solution used was Mouse IgG2a (Dako X0943) diluted to the same concentration as the working antibody solution (0.32 mg/mL). The TRAP primary antibody and isotype antibody incubated on the tissues for 1 hour at room temerature. The primary antibody was then conjugated with a goat anti mouse secondary labelled polymer for 30 minutes at RT (Dako K4001). Staining was then developed with liquid DAB+ for 5 minutes at RT (Dako K3468). Counter staining was developed with automated hematoxylin (S3301) for 10 minutes at RT. The rest of the sections were stained automatically using hematoxylin and eosin (H&E).

Photomicrographs were taken using a Leica Fluorescent Digital Microscope with a (CCD) Digital camera (Leica, Wetzlar, Germany) and the image processing analyses were done using RS Image software (Version 1.73) (Photometrics, Roper Scientific, Inc, Tucson, AZ, USA). Data were collected from three levels along the root: coronal, middle and apical (Figure 6.1). At each level multiple slides were taken and evaluated for the following: odontoblastic layer cell count in each section in three random field/slices (0.001 mm^2), PDL on the four root surfaces were evaluated for cementum thickness, cell count in the PDL, thickness of the PDL, TRAP stained cells on the tooth surface and the bone surface of the socket.



Figur 6.1: Diagram showing a coronal section of the mandible of the animal. The red lines in the diagram indicate the areas where the sections were obtained (C: Coronal, M: Middle and A: Apical).

6.2.3 Blinding and intra-rater reliability measurements:

Each animal's right and left sides were allocated randomly to either the treatment or control group. The same investigator measured the variables from the histological slides three times with a two-week interval between each; measurements were taken on coded samples to test the intra-rater reliability and measurement error. Another investigator coded the samples before starting final data collection.

6.2.4 Statistical Analysis:

Collected data were processed using SPSS 18.0, intra-rater reliability for all the variables were measured using intraclass correlation coefficient (ICC). Due to the small sample size and the split mouth design of the study, we used non-parametric related samples test (Wilcoxon Signed Ranks) to analyze the collected data.

6.3 Results:

6.3.1 Reliability and error:

Intra-rater reliability test results varied among the measured variables where absolute agreement was found in counting the number of TRAP positive cells on the bone and tooth surfaces. On the other hand, it was lower in counting the number of PDL cells (ICC = 0.973) than the odontoblasts cell count (ICC = 0.989). Measurement reproducibility of the cementum thickness and PDL width were very high where the ICC was higher than 0.99.

6.3.2 Cementum:

The histomorphometrical analysis showed significantly thicker cementum on all root surfaces of the teeth with LIPUS compared to the control side. We compared each root surface (mesial, buccal, lingual and distal) of both roots (mesial and distal) to the matching surface on the other side of the mandible. The LIPUS group showed thicker cementum on almost every surface ($P < 0.05$) except for the buccal and mesial surfaces of the distal root cementum at the coronal third ($P = 0.2$ and 0.57, respectively) (Table 6.1 and 6.2). At the coronal third of the root the cementum was acellular and thin. Although the difference was statistically significant between the surfaces at this level, the cementum thickness was small and the mean differences were small (Figure 6.2-A, B, Figure 6.3). The middle third cementum was a mix of mainly acellular cementum and cellular cementum with few cementocytes trapped in lacunae (Figure 6.2- C, D). The mean thickness of cementum on the LIPUS roots middle third was almost two to three times the thickness on control teeth ($P < 0.01$) (Figure 6.4). Finally the apical third cementum was the thickest and mainly cellular (Figure 6.2- E, F). It was double to triple as thick in the LIPUS roots as in the control ones (Figure 6.5).

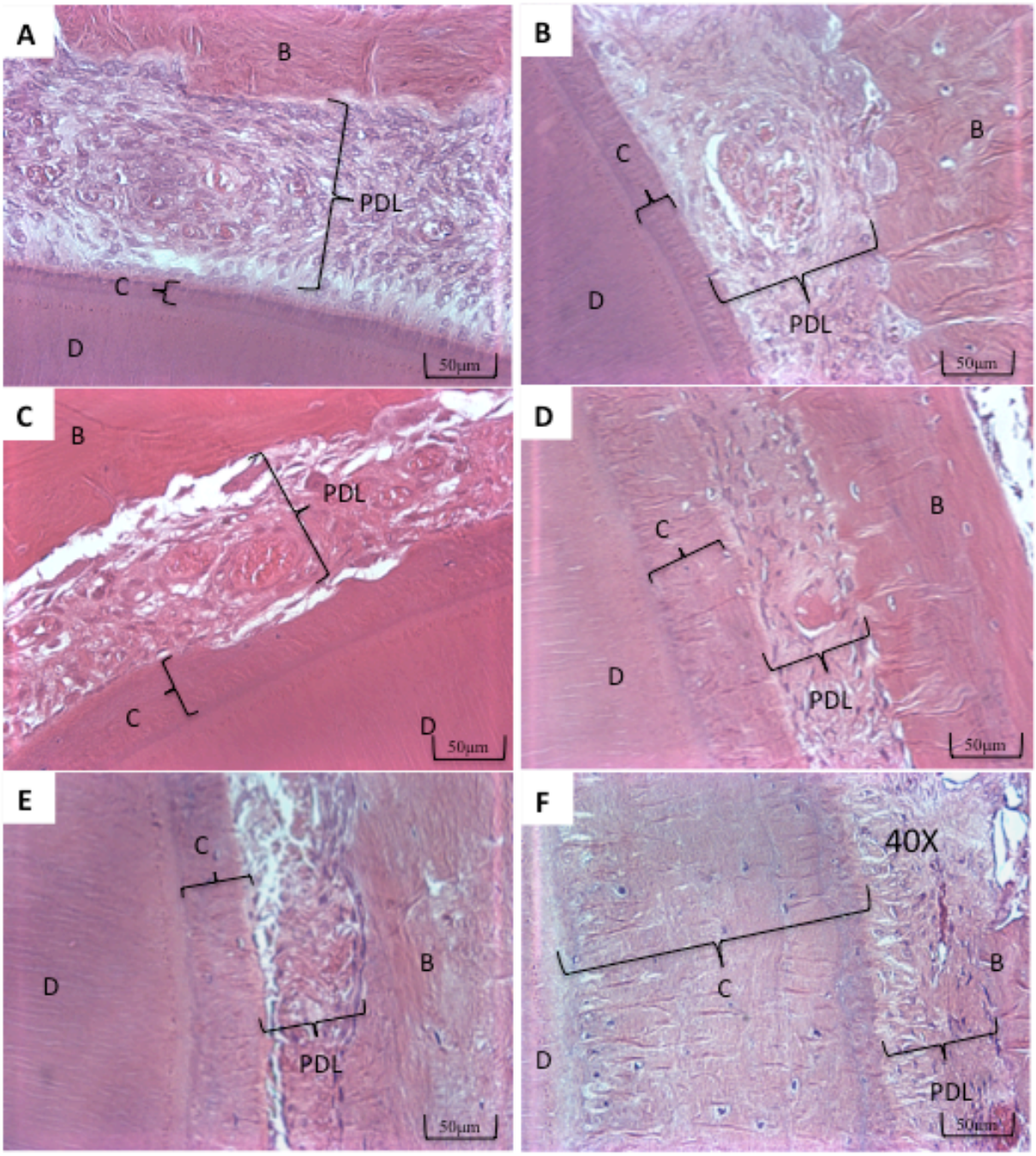


Figure 6.2: H & E stained slides showing the areas of the histomorphometrical analysis in the PDL tissue (40X magnification). A: The root surface of the control tooth at the coronal third with thin acellular cementum and compressed PDL. B: The LIPUS side showing also thin acellular cementum at the coronal third of the root. C & D: The PDL and cementum thickness in the middle third of the root of the control and LIPUS sides, respectively. Showing acellular/cellular cementum thicker than the one in the cervical third. E & F: The PDL and cementum thickness in the apical third of the root of the control and LIPUS sides, respectively. Showing very thick and mainly cellular cementum. (C: Cementum, D: Dentin, PDL: Periodontal ligament, B: Alveolar bone)

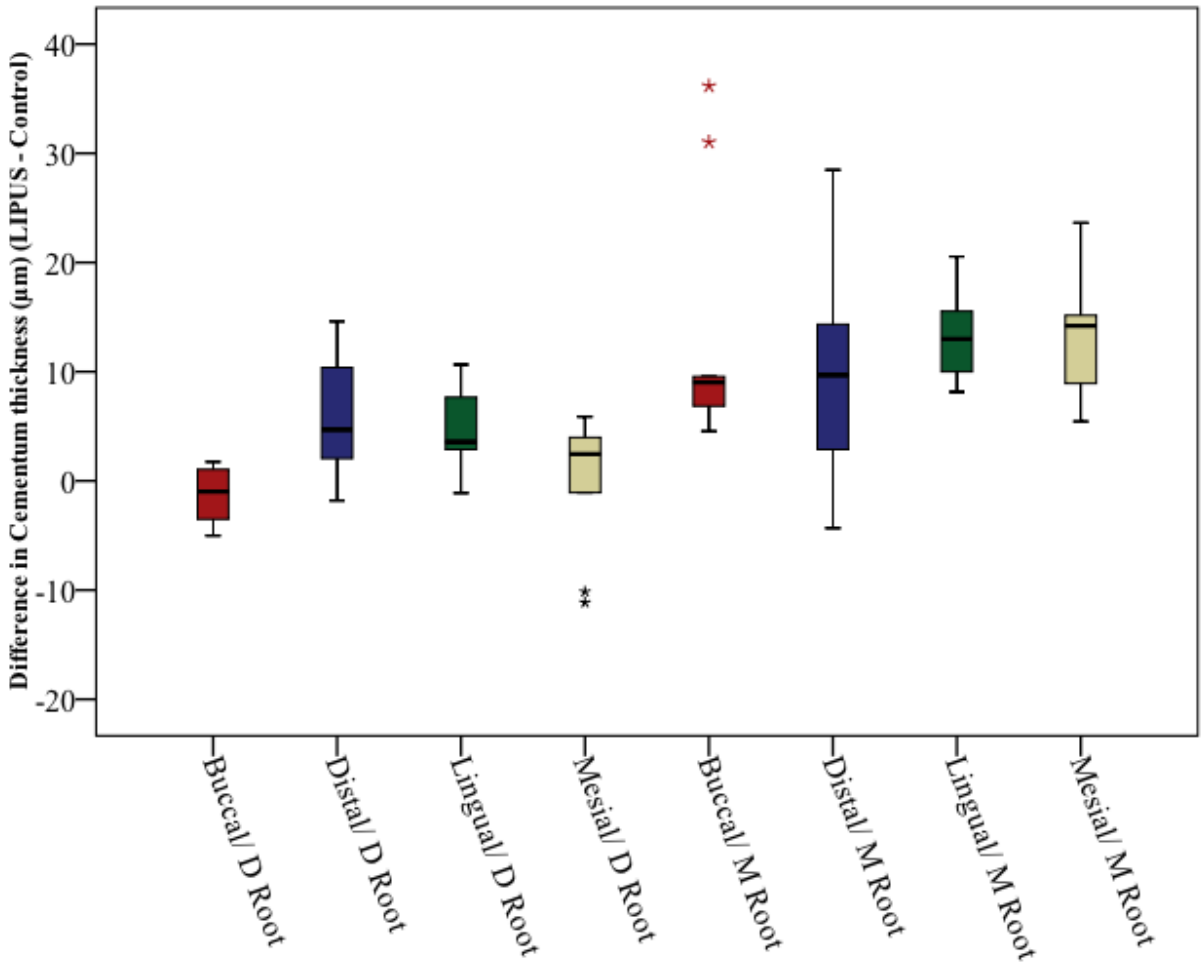


Figure 6.3: Box plot of the difference in cementum thickness (µm) between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side} at the coronal third of the roots on each of the four root surfaces. (X-Axis labels indicate the surface then the root of the mandibular fourth premolar: Surface: Buccal, Mesial, Distal or Lingual/ Root: Distal or Mesial)

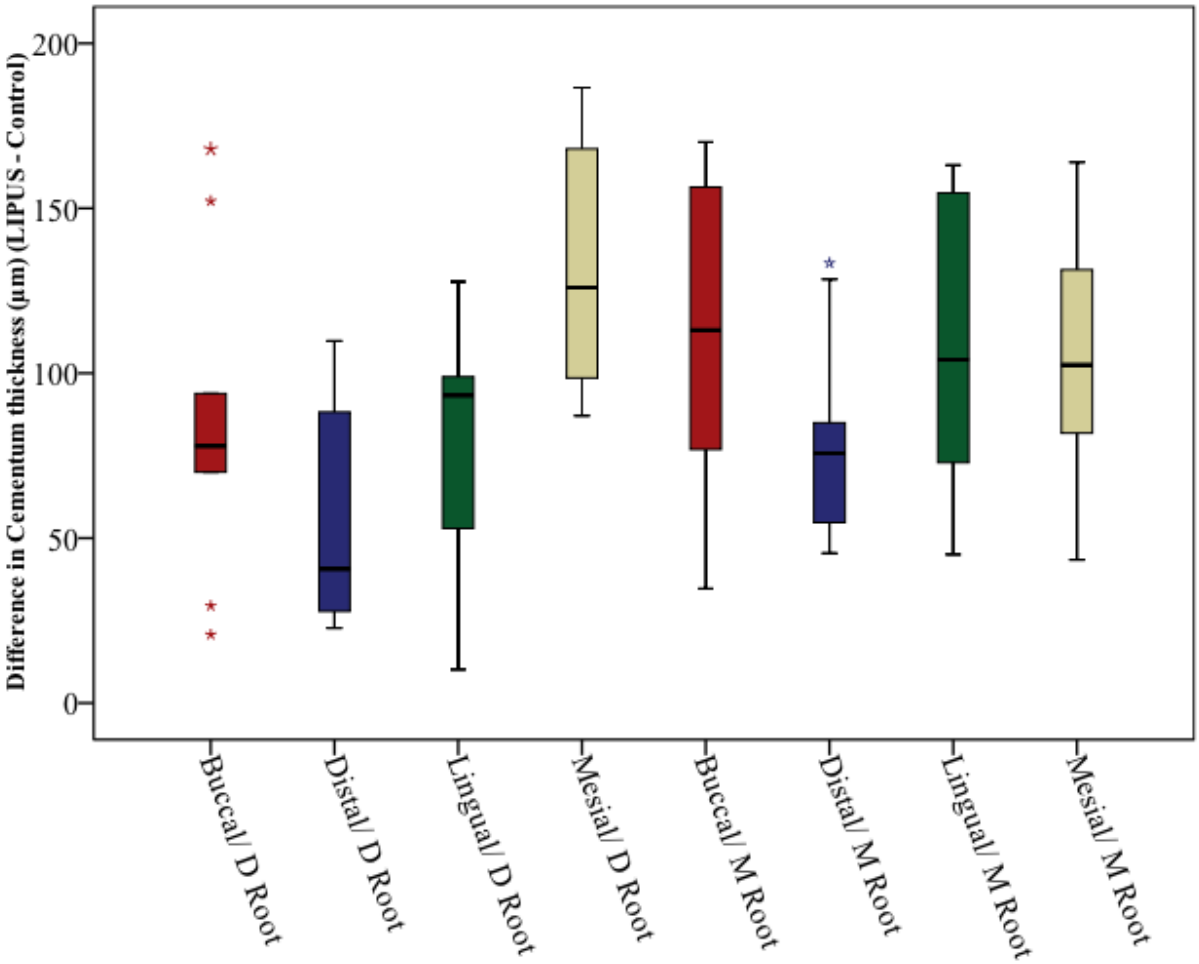


Figure 6.4: Box plot of the difference in cementum thickness (µm) between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side} at the middle third of the roots on each of the four root surfaces. (X-Axis labels indicate the surface then the root of the mandibular fourth premolar: Surface: Buccal, Mesial, Distal or Lingual/ Root: Distal or Mesial)

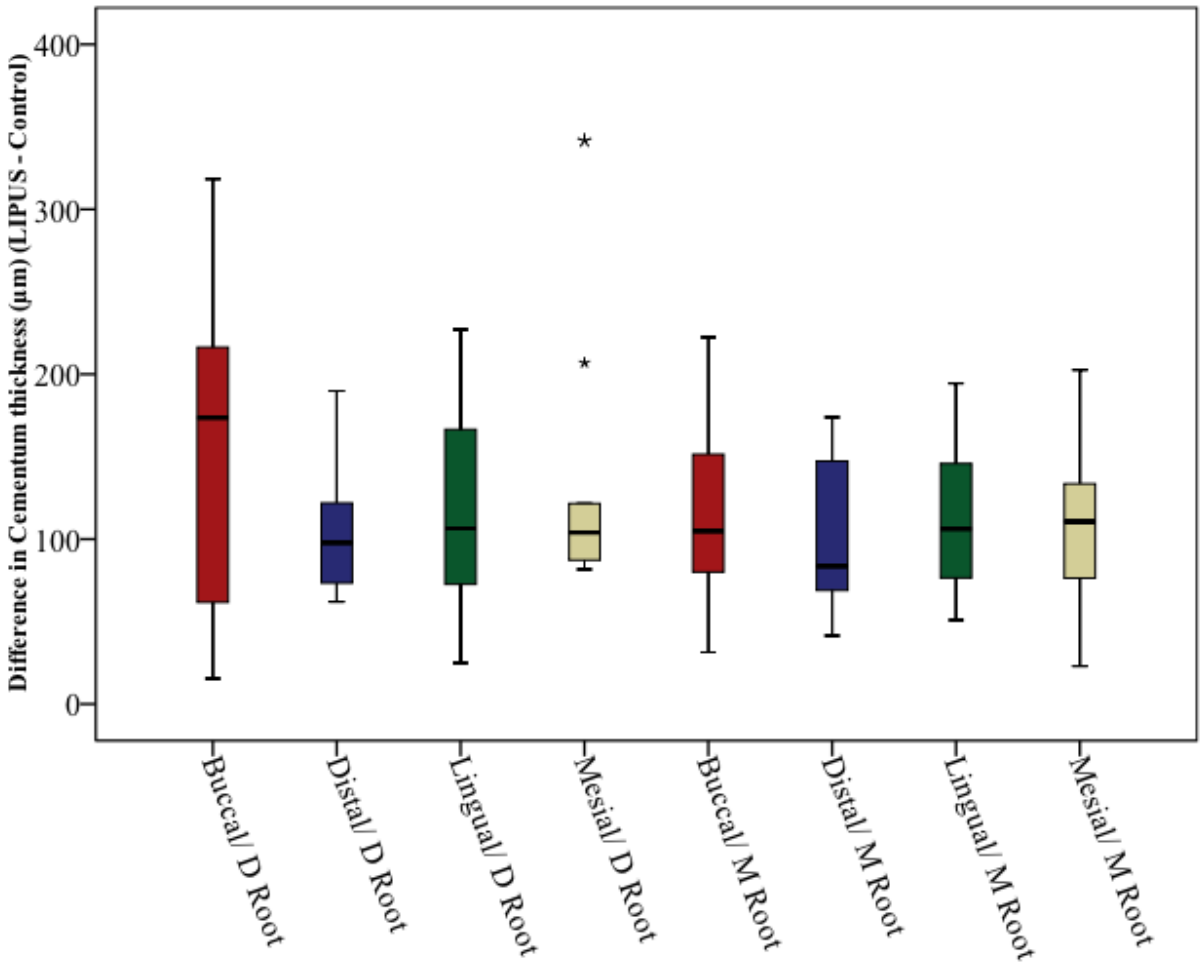


Figure 6.5: Box plot of the difference in cementum thickness (µm) between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side} at the apical third of the roots on each of the four root surfaces. (X-Axis labels indicate the surface then the root of the mandibular fourth premolar: Surface: Buccal, Mesial, Distal or Lingual/ Root: Distal or Mesial)

6.3.3 Periodontal ligament:

The mean width of the PDL ranged from 66 to 215 μm at all four root surfaces and at the three levels measured. The Wilcoxon Signed Ranks test showed only a few areas in which the statistical difference was significant. The control PDL was wider than the LIPUS group on the mesial surface of the distal and mesial roots (P = 0.02, 0.005, respectively) but narrower on the distal surface of the mesial root (P <0.01); all were at the coronal level of the roots (Table 6.1 and 6.2, Figures 6.6-6.8).

Furthermore, the cellularity of the PDL in the LIPUS group was higher than the control group in most of the examined areas around the root (both compression or tension side). The difference was statistically significant (P < 0.05) in all areas except on the mesial PDL of the distal root middle third (P = 0.059) and the buccal PDL of the mesial root apical third (P = 0.154). The cell count mean difference ranged from 20 to 50% more in the LIPUS group (Table 6.1 and 6.2, Figures 6.9-6.11).

Table 6.1: The mean and standard deviations of the cementum thickness, PDL thickness and PDL cell count variables on the four surfaces of the mesial and distal roots of the fourth premolar. (B: Buccal surface, M: Mesial surface, D: Distal surface, L: Lingual surfaces, M: mean, SD: standard deviation, PDL: periodontal ligament)

			Cementum thickness (µm)		PDL thickness (µm)		PDL cells	
			LIPUS	CTL	LIPUS	CTL	LIPUS	CTL
			M (SD)	M (SD)	M (SD)	M (SD)	M (SD)	M (SD)
Coronal 1/3	Distal Root	B	11.76 (2.93)	13.03 (3.06)	127.99 (44.39)	140.18 (28.31)	6.03 (1.2)	3.94 (0.77)
		D	21.07 (6.04)	15.85 (2.67)	163.4 (52.36)	153.69 (20.57)	4.42 (1.05)	3.245 (0.57)
		L	19.44 (6.85)	14.93 (4.53)	121.71 (46.89)	120.52 (26.78)	5.87 (2.12)	3.27 (0.85)
		M	18.15 (6.13)	18.15 (7.07)	66.99 (42.47)	113.21 (28.74)	7.07 (1.71)	3.98 (0.84)
Middle 1/3		B	108.57 (46.04)	23.35 (19.53)	110.72 (54.92)	124.41 (37.8)	6.03 (1.43)	4.15 (0.61)
		D	93.86 (50.48)	36.08 (18.53)	126.06 (29.23)	111.35 (20.9)	6.17 (2.36)	3.07 (0.87)
		L	113.55 (40.86)	36.91 (12.5)	121.87 (47.54)	107.81 (26.93)	7.19 (1.31)	3.85 (0.67)
		M	175.74 (33.96)	41.87 (15.25)	91.97 (55.21)	112.05 (46.84)	6.98 (3.13)	4.15 (0.69)
Apical 1/3		B	192.45 (91.94)	81.1 (37.49)	206.1 (101.64)	140.04 (53.32)	5.48 (1.15)	3.28 (0.74)
		D	190.14 (54.64)	81.12 (31.58)	212.34 (78.88)	138.37 (51.96)	4.91 (1.28)	3.21 (0.75)
		L	171.05 (81.27)	55.67 (31.07)	188.93 (92.67)	129.01 (70.88)	5.17 (0.83)	3.25 (0.57)
		M	208.61 (77.96)	74.81 (33.48)	194.21 (118.22)	113.28 (59.19)	5.98 (1.43)	2.78 (1.12)
Coronal 1/3	Mesial Root	B	24.17 (12.19)	11.4 (3.58)	112.86 (44.52)	110.51 (28.72)	7.24 (1.18)	3.04 (0.76)
		D	25.96 (11.44)	15.76 (6.77)	99.65 (13.47)	75.4 (9.08)	4.87 (1.76)	3.16 (0.41)
		L	27.02 (6.49)	13.91 (3.76)	94.97 (46.86)	180.43 (164.78)	5.81 (1.16)	2.505 (1.39)
		M	26.64 (7.16)	13.29 (2.5)	73.13 (29.31)	144.99 (63.84)	5.72 (1.89)	3.77(0.74)
Middle 1/3		B	140.6 (43.82)	30.73 (12.08)	163.11 (85.94)	114.14 (32.65)	5.62 (2.64)	3.66 (1.01)
		D	119.22 (26.57)	39.64 (19.69)	97.17 (23.18)	104.1 (63.76)	5.55 (1.3)	3 (0.46)
		L	141.48 (40.91)	32.59 (14.13)	105.14 (36.25)	94.16 (33.26)	6.73 (1.56)	3.39 (0.48)
		M	133.65 (41.66)	29.89 (17.6)	132.36 (60.8)	109.97 (36.51)	5.78 (1.07)	3.27 (0.54)
Apical 1/3		B	164.13 (52.3)	42.68 (18.96)	185.52 (105.16)	215.97 (123.66)	4.15 (1.36)	3.29 (1.46)
		D	150.32 (34.22)	49.45 (19.33)	172.97 (103.24)	182.33 (128.05)	5.65 (0.52)	3.29 (1.22)
		L	160.82 (36.9)	48.06 (13.4)	160.17 (86.36)	210.66 (102.4)	8.3 (1.38)	3.69 (1.18)
		M	164.35 (53.83)	56.12 (13.91)	157.98 (49.77)	205.04 (74.38)	5.83 (1.26)	3.21 (1.07)

Table 6.2: The results of non-parametric test (Wilcoxon Signed Ranks test) for comparing the three variables (Cementum thickness, PDL width and cell count) on the four surfaces of the mesial and distal roots of the fourth premolar. {MD: Mean difference, P: P-value}

		Buccal/ Distal root		**Distal/ Distal root**		**Lingual/ Distal root**		**Mesial/ Distal root**		**Buccal/ Mesial root**		**Distal/ Mesial root**		**Lingual/ Mesial root**		**Mesial/ Mesial root**	
		MD	**P**	**MD**	**P**	**MD**	**P**	**MD**	**P**	**MD**	**P**	**MD**	**P**	**MD**	**P**	**MD**	**P**
Cementum Thickness (µm)	**Corona 1 1/3**	1.27	0.203	5.21	0.013*	4.51	0.013*	0.0	0.575	12.77	<0.01*	10.2	0.017*	13.1	<0.01*	13.35	<0.01*
	Middle 1/3	85.2	<0.01*	57.78	<0.01*	76.63	<0.01*	133.86	<0.01*	109.87	<0.01*	79.58	<0.01*	108.88	<0.01*	103.75	<0.01*
	Apical 1/3	111.34	<0.01*	109.02	<0.01*	115.38	<0.01*	133.79	<0.01*	121.45	<0.01*	100.86	<0.01*	112.76	<0.01*	108.23	<0.01*
PDL Width (µm)	**Corona 1 1/3**	12.19	0.575	9.7	0.445	1.19	0.799	46.21	0.022*	2.34	0.721	24.25	<0.01*	85.45	0.24	71.85	<0.01*
	Middle 1/3	13.68	0.508	14.7	0.241	14.06	0.508	20.07	0.445	48.96	0.386	6.93	0.959	10.98	0.445	22.39	0.386
	Apical 1/3	66.06	0.169	73.96	0.59	59.91	0.285	80.92	0.203	30.45	0.169	9.36	0.386	50.49	0.074	47.05	0.059
PDL cell count	**Corona 1 1/3**	2.09	<0.01*	1.17	0.036*	2.6	0.012*	3.1	<0.01*	4.2	<0.01*	1.71	0.012	3.3	<0.01*	1.95	0.012
	Middle 1/3	1.88	<0.01*	3.1	<0.01*	3.34	<0.01*	2.83	0.059	1.96	0.036*	2.55	<0.01*	3.34	<0.01*	2.51	<0.01*
	Apical 1/3	2.2	0.012*	1.7	<0.01*	1.92	<0.01*	3.2	<0.01*	0.86	0.154	2.36	<0.01*	4.61	<0.01*	2.62	<0.01*

Table 6.3: The mean and standard deviations of the odontoblast cell count, TRAP stained cells attached to the bone and tooth and their percentage at the three examined root levels. (OD: odontoblast cells)

	OD count				**Bone TRAP count**				**Tooth TRAP**				**T-B % TRAP**			
	LIPUS		**CTL**		**LIPUS**		**CTL**		**LIPUS**		**CTL**		**LIPUS**		**CTL**	
	M	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
Coronal 1/3	11.2	1.37	6.43	2.02	16.4	3.06	15.8	2.85	0.1	0.31	1.4	0.51	0.47	1.5	9.18	3.98
Middle 1/3	12.75	2.08	5.41	0.91	34.7	4.49	27.7	4.59	1	0.81	3.7	1.15	2.85	2.18	13.2	2.67
Apical 1/3	6.81	0.92	6.13	0.97	18.1	3.1	15.8	2.57	0.7	0.67	3	1.15	3.79	3.88	19.02	6.9

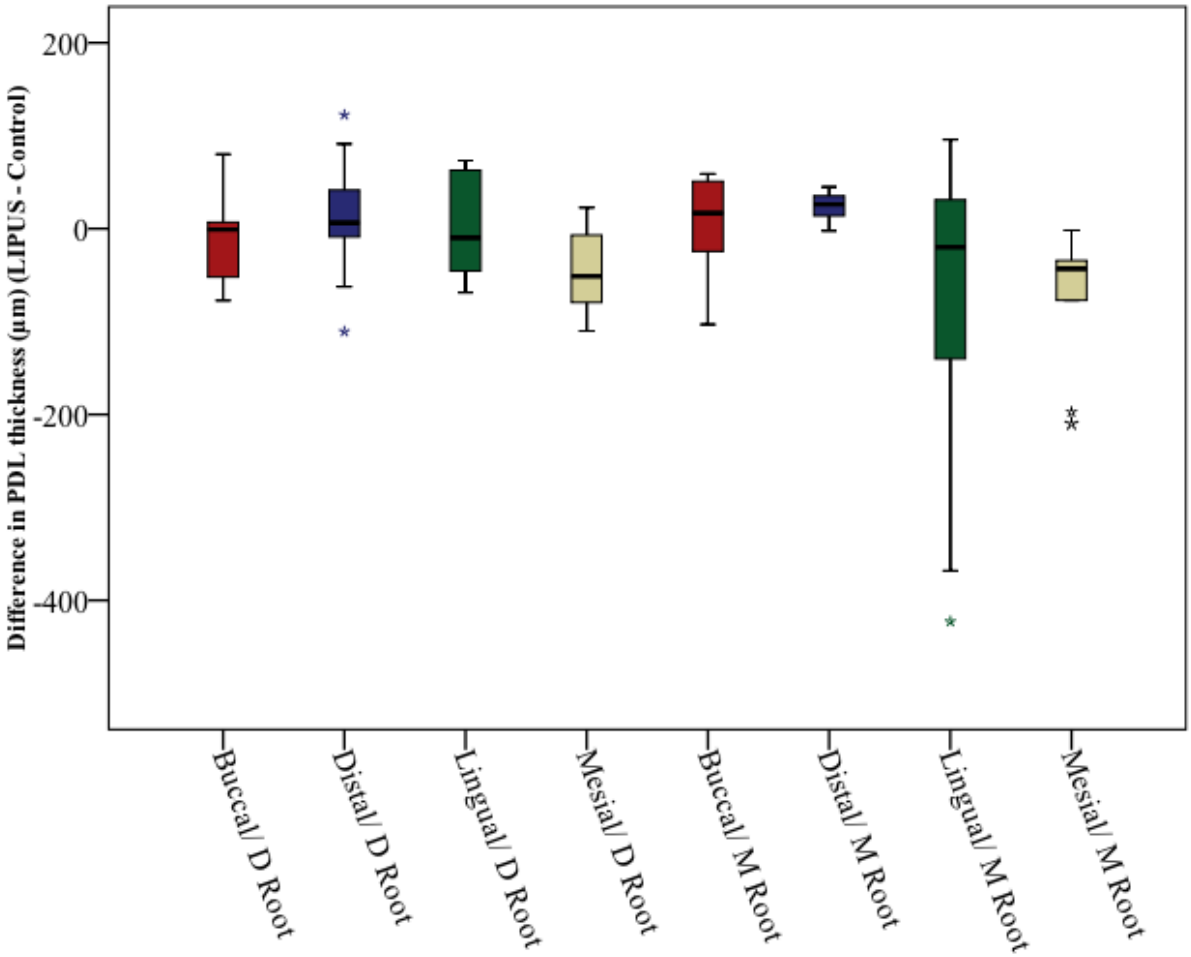


Figure 6.6: Box plot of the difference in PDL thickness (µm) between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side} at the coronal third of the roots on each of the four root surfaces. (X-Axis labels indicate the surface then the root of the mandibular fourth premolar: Surface: Buccal, Mesial, Distal or Lingual/ Root: Distal or Mesial)

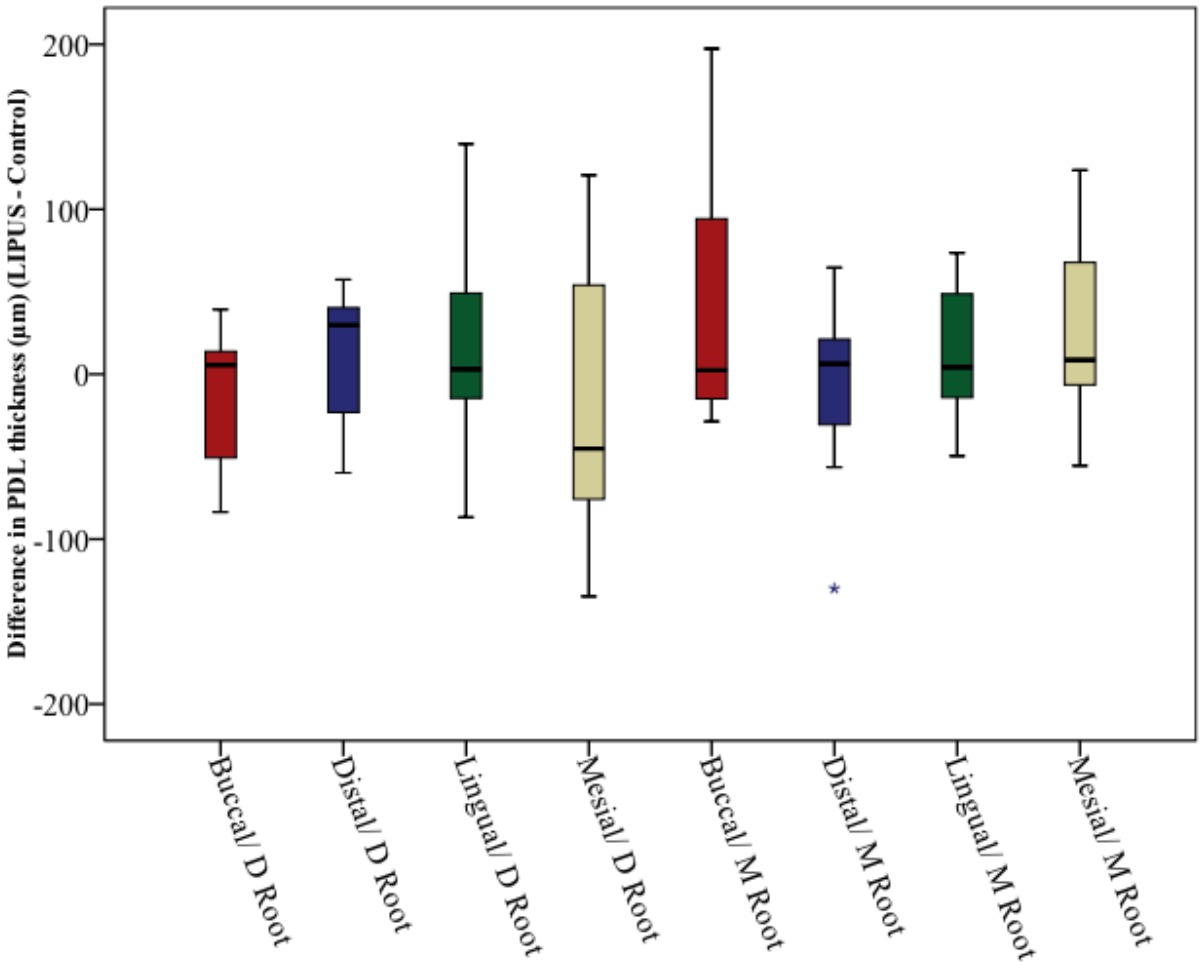


Figure 6.7: Box plot of the difference in PDL thickness (µm) between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side} at the middle third of the roots on each of the four root surfaces. (X-Axis labels indicate the surface then the root of the mandibular fourth premolar: Surface: Buccal, Mesial, Distal or Lingual/ Root: Distal or Mesial)

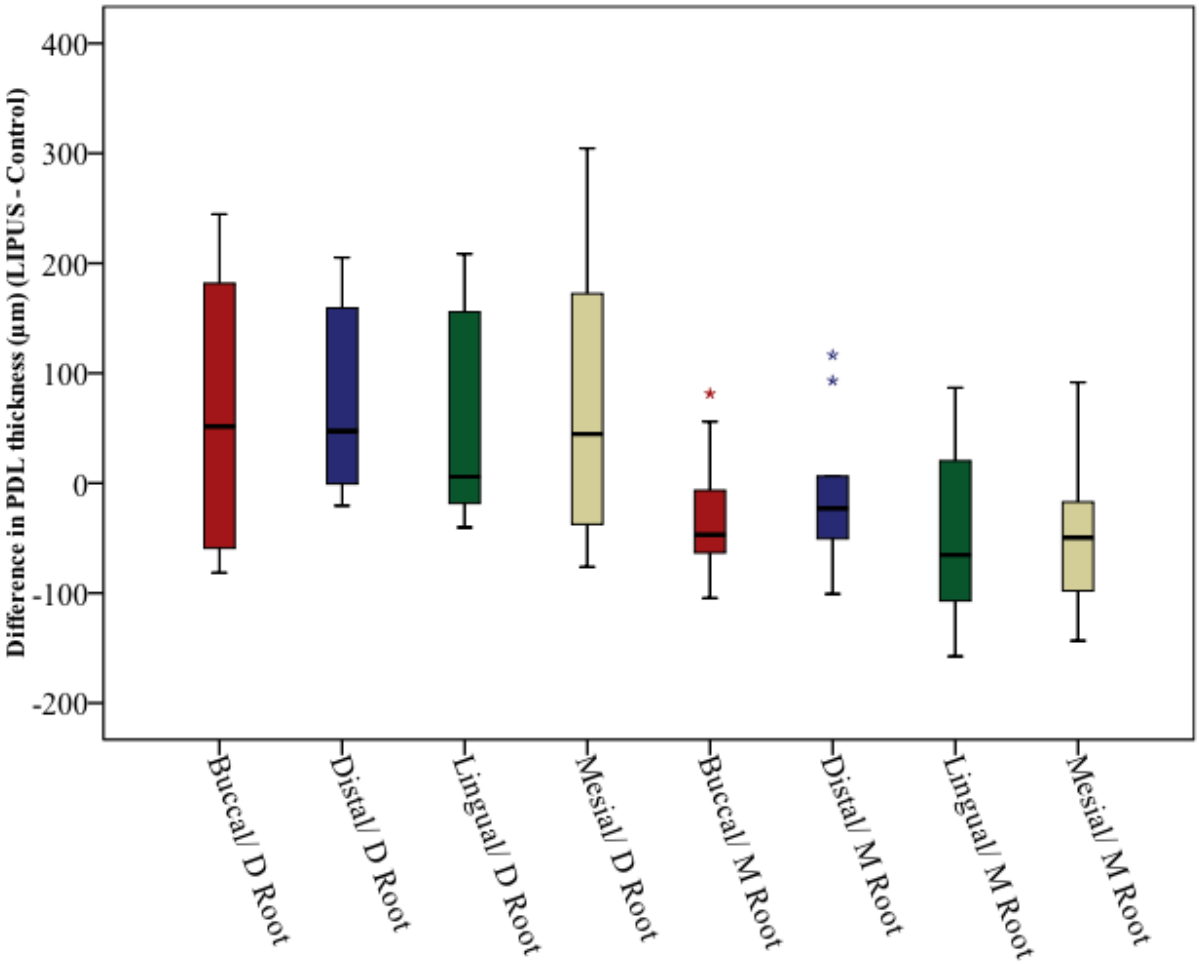


Figure 6.8: Box plot of the difference in PDL thickness (µm) between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side} at the apical third of the roots on each of the four root surfaces. (X-Axis labels indicate the surface then the root of the mandibular fourth premolar: Surface: Buccal, Mesial, Distal or Lingual/ Root: Distal or Mesial)

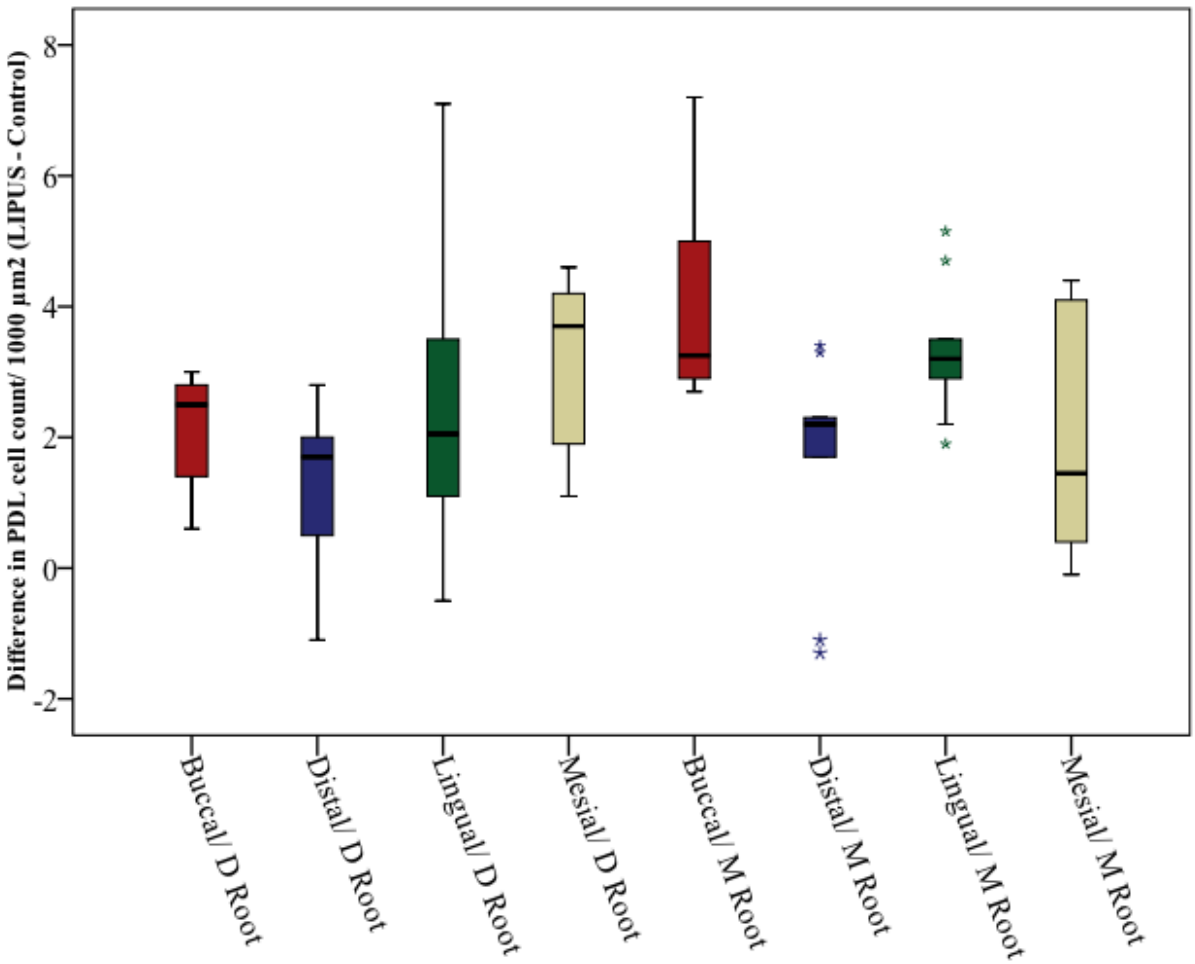


Figure 6.9: Box plot of the difference in cell count in the PDL between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side} around the coronal third of the root on each of the four root surfaces in the control and LIPUS sides. (Graph key indicates the surface then the root of the mandibular fourth premolar: Surface: Buccal, Mesial, Distal or Lingual/ Root: Distal or Mesial)

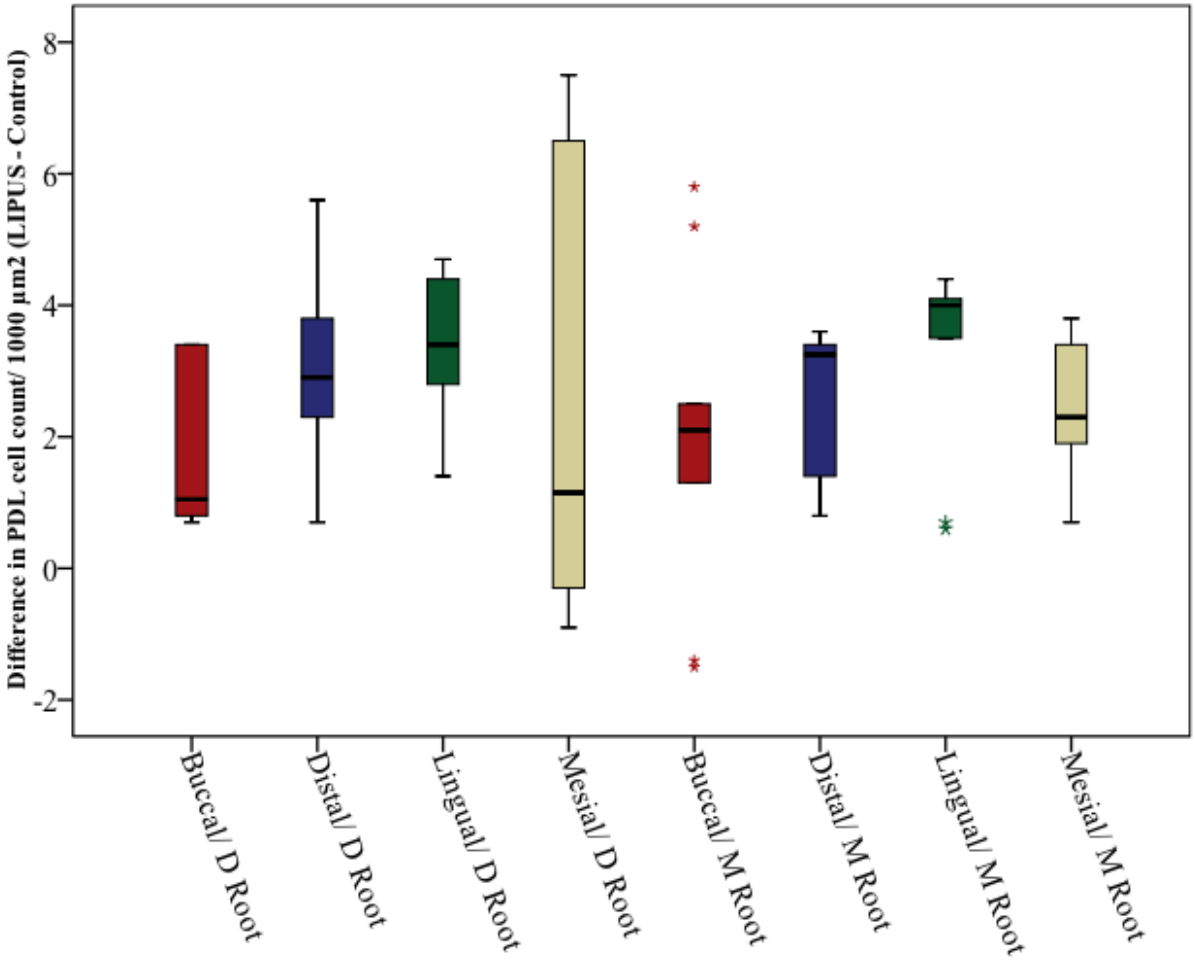


Figure 6.10: Box plot of the difference in cell count in the PDL between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side} around the middle third of the root on each of the four root surfaces in the control and LIPUS sides. (Graph key indicates the surface then the root of the mandibular fourth premolar: Surface: Buccal, Mesial, Distal or Lingual/ Root: Distal or Mesial)

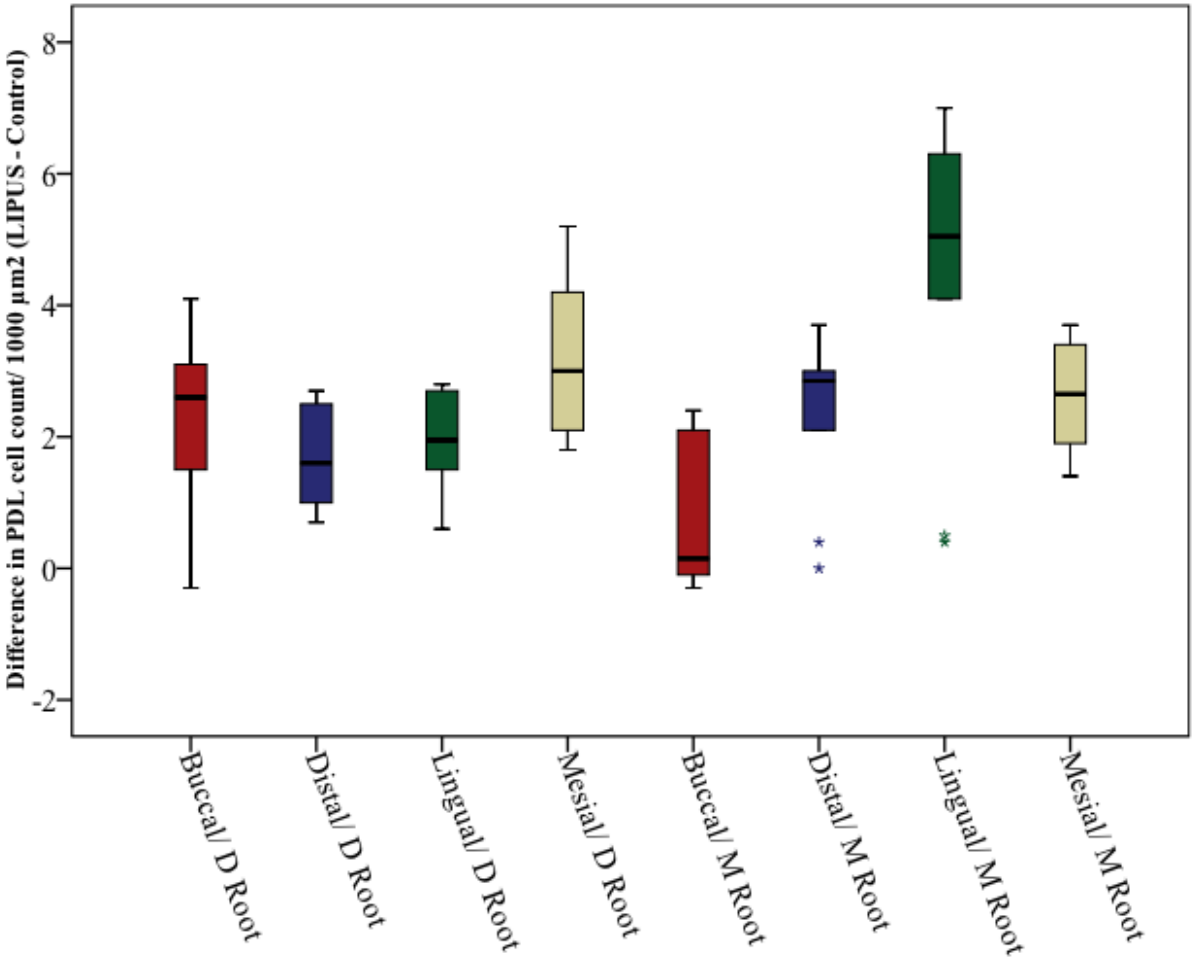


Figure 6.11: Box plot of the difference in cell count in the PDL between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side} around the apical third of the root on each of the four root surfaces in the control and LIPUS sides. (Graph key indicates the surface then the root of the mandibular fourth premolar: Surface: Buccal, Mesial, Distal or Lingual/ Root: Distal or Mesial)

6.3.4 *Dental pulp:*

From the H & E stained slides, we found that beagle dog's dental pulp does not have a predentin layer beneath the odontoblastic cell layer, also the pulp core is highly vascular in both the control and LIPUS groups (Figure 6.12). The odontoblastic cell layer is well defined with the normal columnar shape cells attached to the dentin layer in the coronal and middle third of the root (Figure 6.12 A, B, C & D), but it was less defined in the apical third (Figure 6.12 E &F). The LIPUS teeth odontoblasts cell count was higher in the coronal and middle thirds of the root by a mean difference of 80% and 100% more than the control teeth ($P < 0.01$), but there was no statistically significant difference between the apical third cell counts ($P = 0.169$) (Figure 6.13, Table 6.3 and 6.4)

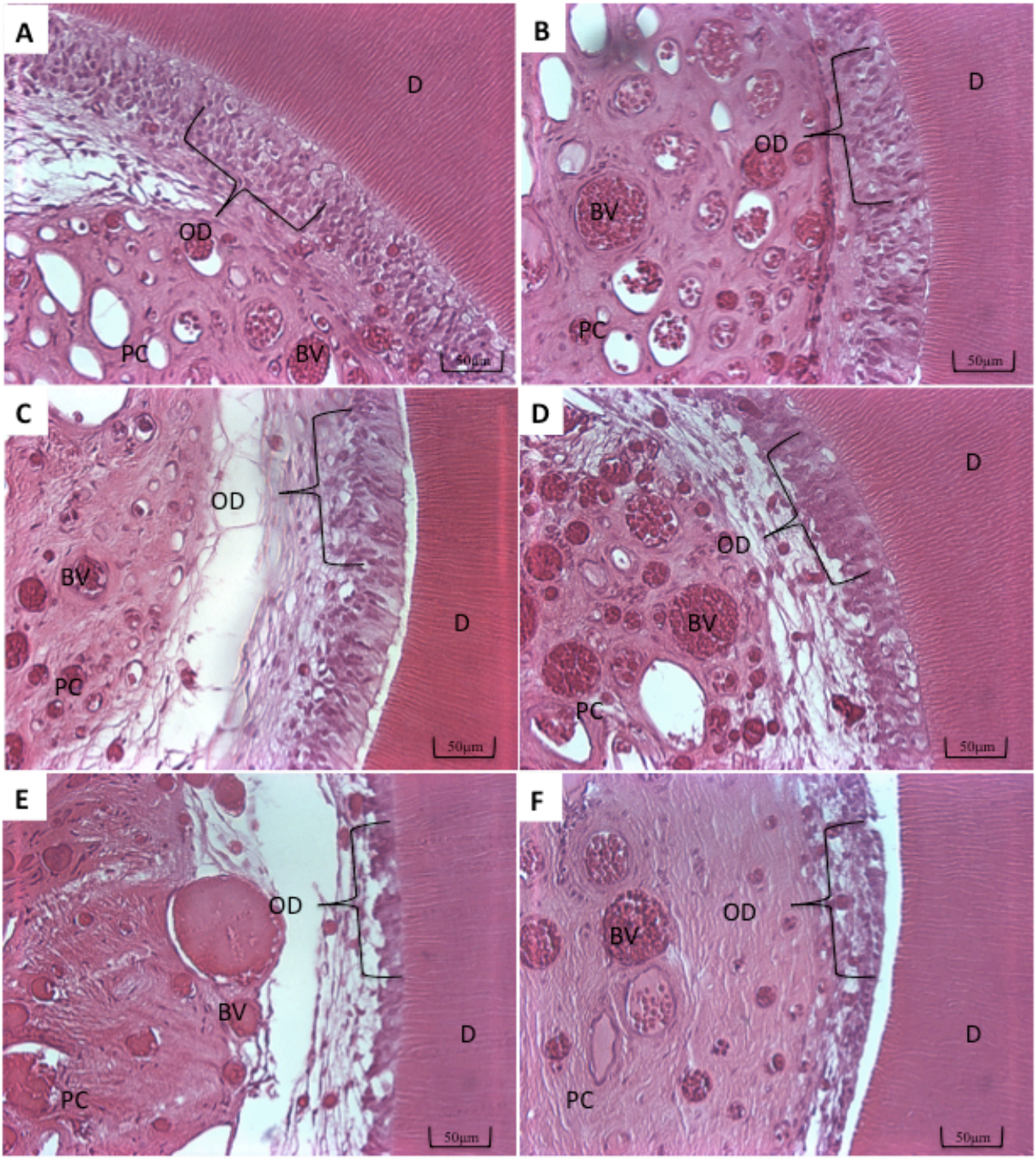


Figure 6.12: H & E stained slides showing the areas of the histomorphometrical analysis in the dental pulp (40X magnification). A: Control, B: LIPUS teeth dental pulp at the coronal third of the root showing intact and normal odontoblastic cell layer with no predentin layer. C & D: The PDL and cementum thickness in the middle third of the root of the control and LIPUS sides, respectively. Showing acellular/cellular cementum thicker than the one in the cervical third. E & F: The PDL and cementum thickness in the apical third of the root of the control and LIPUS sides, respectively. Showing very thick and mainly cellular cementum. (OD: Odontoblasts, D: Dentin, PC: Pulp core, BV: Blood vessel)

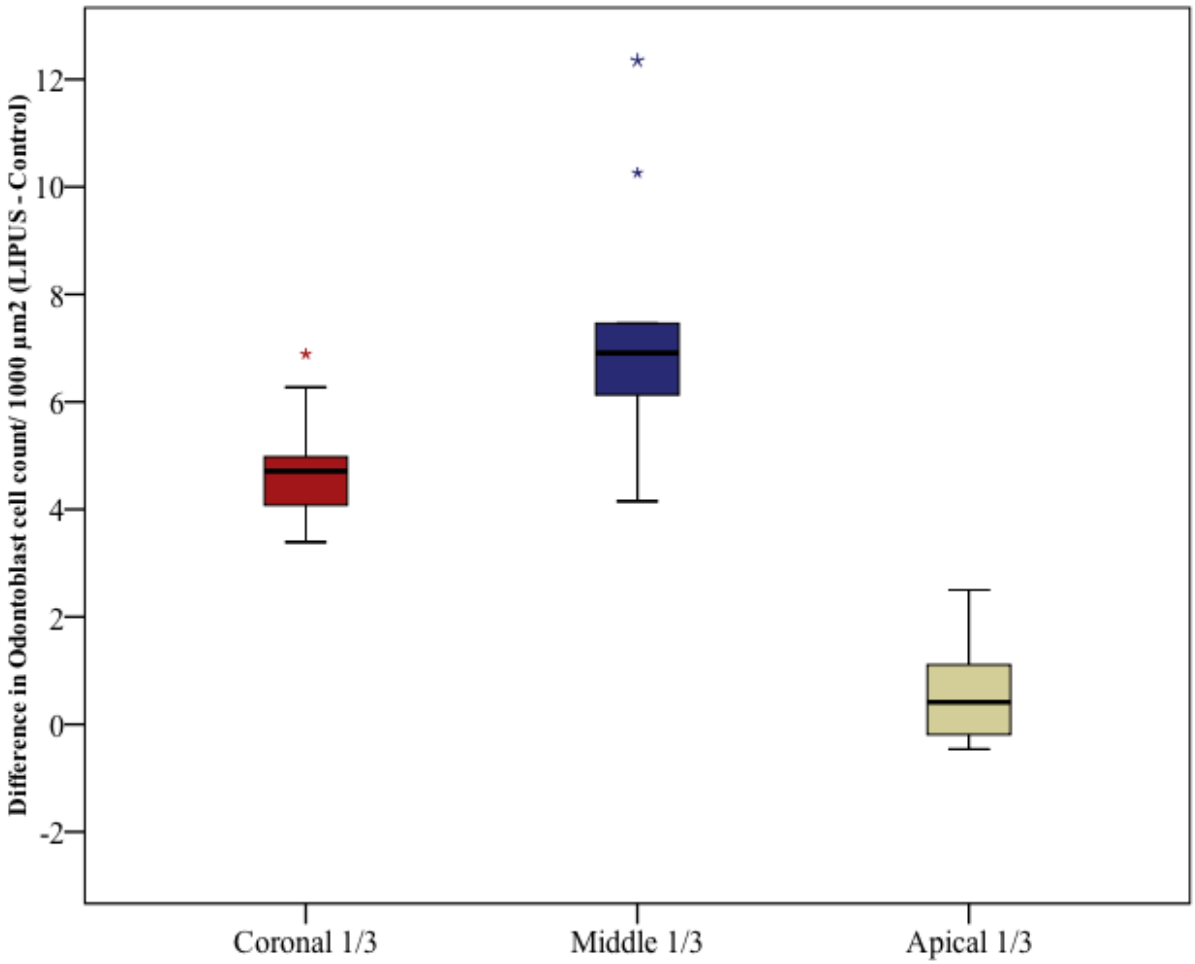


Figure 6.13: Box plot of the difference in odontoblast cell count/1000 µm^2 between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side} at the three levels of the root.

6.3.5 Root resorption:

Due to the difficulty and subjective nature of evaluating and assessing the quantity of root resorption histologically and the inability to measure it in volumetric terms, we are only presenting descriptive findings about root resorption as seen in the histological slides. The root resorption lacunae were more severe and extended beyond the cementum into the underlying dentin in the control teeth (Figure 6.14 A, C), while they were less severe and to some extent milder with the presence of irregularly formed cellular cementum (reparative cementum) deposited in the resorption lacunae (Figure 6.14 B, D). Also, in some LIPUS roots we found only on the buccal surface a sudden change in the thickness of the cementum layer (Figure 6.14 F). Furthermore, on the middle and apical thirds of the LIPUS roots we found a thin layer of pre-cementum (Figure 6.14 H) but not in the control group. In both groups we found a very close association between areas of root resorption and frontal bone resorption and blood vessels in the PDL (Figure 19).

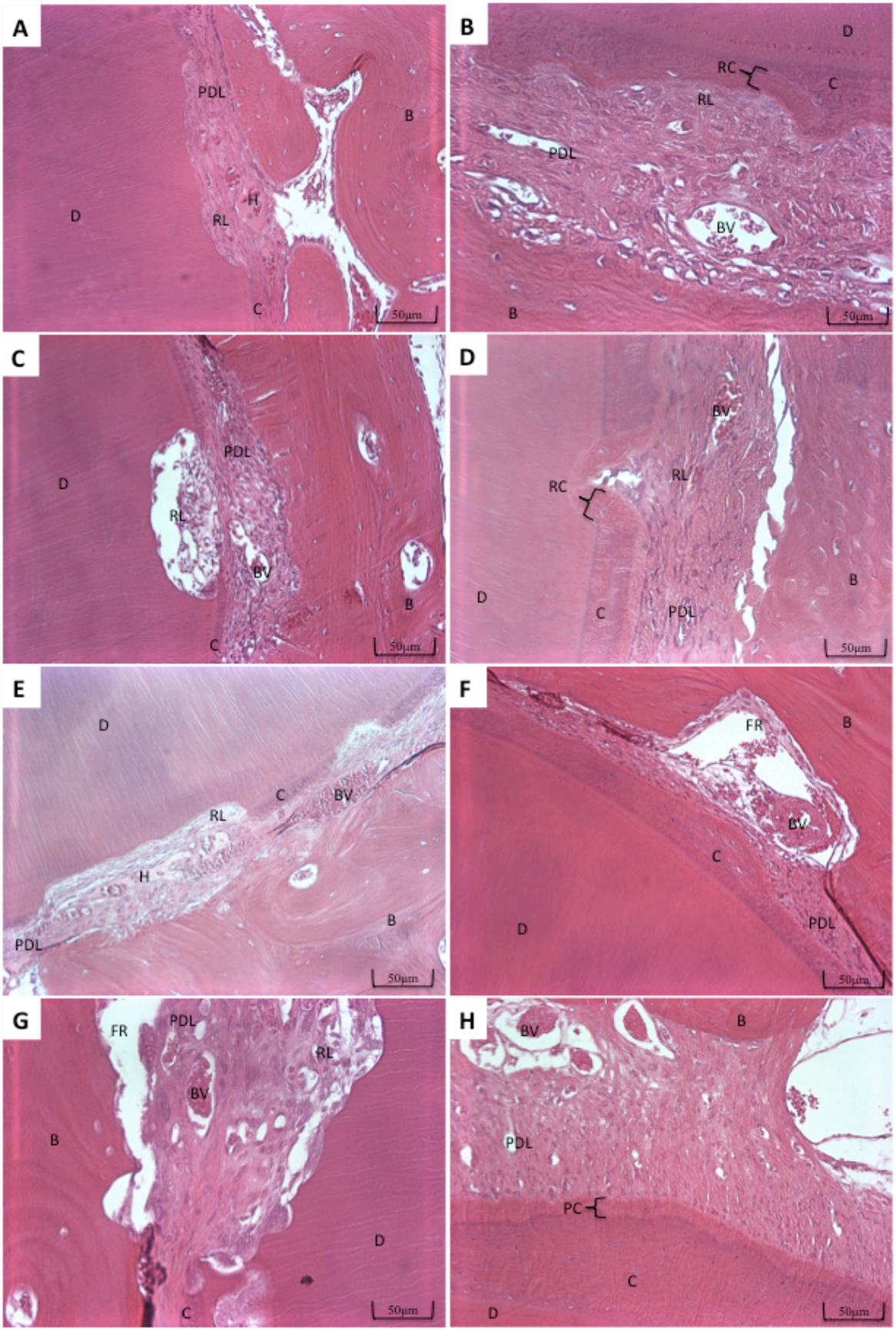

A
PDL
B
H
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Figure 6.14: H & E stained slides showing areas of root resorption and repair process in the LIPUS group compared to the control (40X magnification). A & C: Control root PDL showing an area of hyalinization (H) of the tissue adjacent to the resorption lacuna (RL) that extends beyond the cementum into the dentin, B & D: LIPUS root showing the apposition of irregular cellular cementum into the RL which extends into the cementum and sometimes into the dentin. E & G: Root resorption lacunae (RL) in the control teeth showed more aggressive and deep lacunae, also it has been observed in association with hyalinization areas and more commonly with a blood vessel in the PDL. Also alveolar bone frontal resorption was found to co-exist sometimes with root resorption area. F & H: In some of the animals LIPUS roots, a sudden change in the thickness of cementum were found on the buccal side. Also pre-cementum layer was evident in the middle and apical third of the roots of the LIPUS side only. (PDL: Periodontal ligament, C: Cementum, RC: Repair cementum, PC: Pre-cementum, D: Dentin, RL: Root resorption lacuna, FR: Alvolar bone frontal resorption, H: Hyalinization of the PDL, BV: Blood vessel)

Table 6.4: The results of multiple comparisons (Tamhane's test) between groups for the variables odontoblast count (OD) and TRAP stained cells attached to the bone, tooth and the percentage between them. (CTL: control, Dif: mean difference, SE: standard error, P: p-value *: Statistically significant).

	Coronal region Level A (Coronal 1/3)		Middle region Level B (Middle 1/3)		Apical region Level C (Apical 1/3)	
	MD (SD)	P-value	MD (SD)	P-value	MD (SD)	P-value
Odontoblast cell count	4.76 (1.11)	<0.01*	7.34 (2.36)	<0.01*	0.67 (1.1)	0.169
TRAP cells on bone	0.6 (1.4)	0.156	7 (3.6)	<0.01*	2.3 (2.8)	0.041*
TRAP cells on tooth	1.3 (0.48)	<0.01*	2.7 (0.67)	<0.01*	2.3 (0.67)	<0.01*
Tooth/Bone TRAP percentage	8.7 (4)	<0.01*	10.35 (1.3)	<0.01*	15.22 (4.58)	<0.01*

6.3.6 TRAP immunohistochemistry:

From the Tartrate-Resistant Acid Phosphatase (TRAP) immunohistochemistry we were able to map out the pattern of odontoclasts and osteoclasts present in the PDL (Figure 6.15). We found that the TRAP positive cells on the bone surface (osteoclasts) were not different at the coronal third of the root ($P = 0.156$) but higher in the LIPUS group at the middle third by 25% and at the apical third by 11% ($P = 0.006$, 0.04 respectively) (Table 6.3 and 6.4) (Figure 6.16). However, the TRAP positive cells on the tooth surface (Odonto/cementoclasts) were higher on the control teeth at the three levels compared to the LIPUS teeth ($P < 0.01$) (Table 6.3 and 6.4) (Figure 6.17). Among the three levels, the TRAP positive odonto/cementoclasts were lowest in the coronal third of the root in both the control and the LIPUS groups. Finally, we calculated the percentage of the odonto/cementoclasts to osteoclasts at each level and we found that the percentage at all levels higher in the control group ($P < 0.01$) (Table 6.3 and 6.4). Also the percentages tend to increase when we go apical in both groups with the coronal third being the lowest (Figure 6.18).

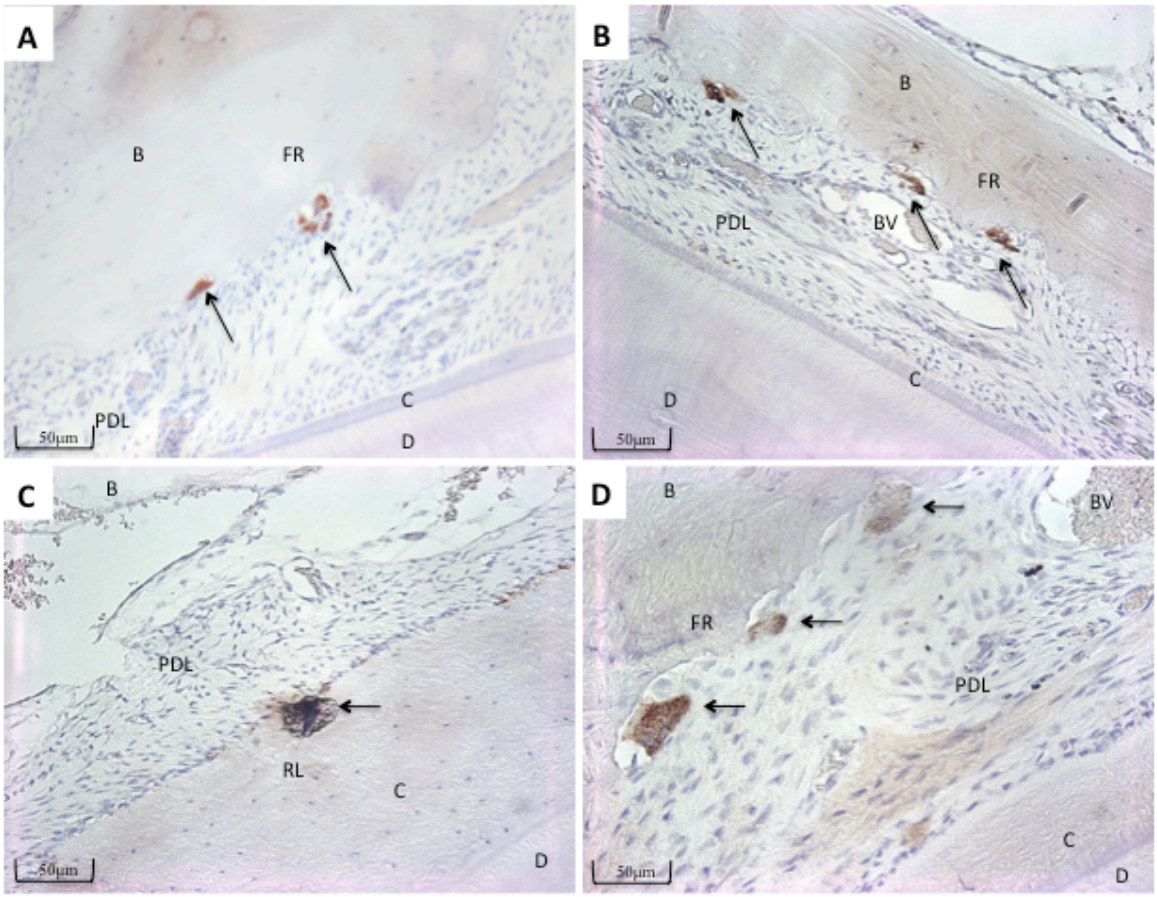

Figure 6.15: TRAP immunohistochemistry: TRAP positive cell cytoplasm was stained brown as shown by the *arrows*. A: Area of PDL with shear forces across the ligaments due to the orientation of the fibres, osteoclasts are present in their lacunae on the bone surface. B: PDL compression area with compressed blood vessels and osteoclasts on the bone surface. C: TRAP positive odonto/cementoclast in RL on the cementum. D: Osteoclasts close to a large blood vessel in the PDL of LIPUS teeth with highly cellular PDL. (PDL: Periodontal ligament, C: Cementum, D: Dentin, RL: Root resorption lacuna, FR: Alveolar bone frontal resorption, BV: Blood vessel)

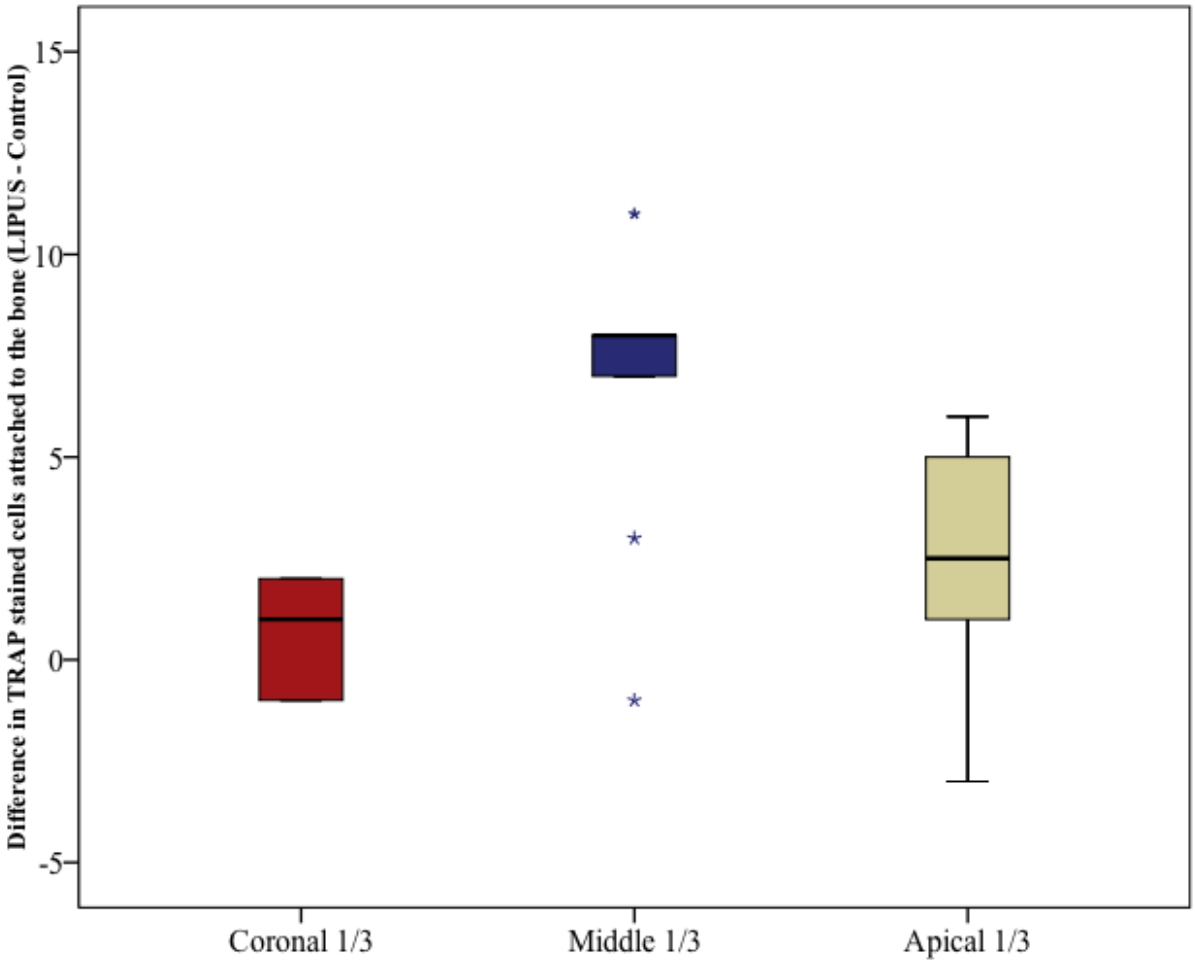

Figure 6.16: Box plot of the difference in TRAP positive cell count attached to the alveolar bone surface inside the PDL (osteoclasts) between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side}, showing that it was highest at the middle third of the root in the control and LIPUS teeth.

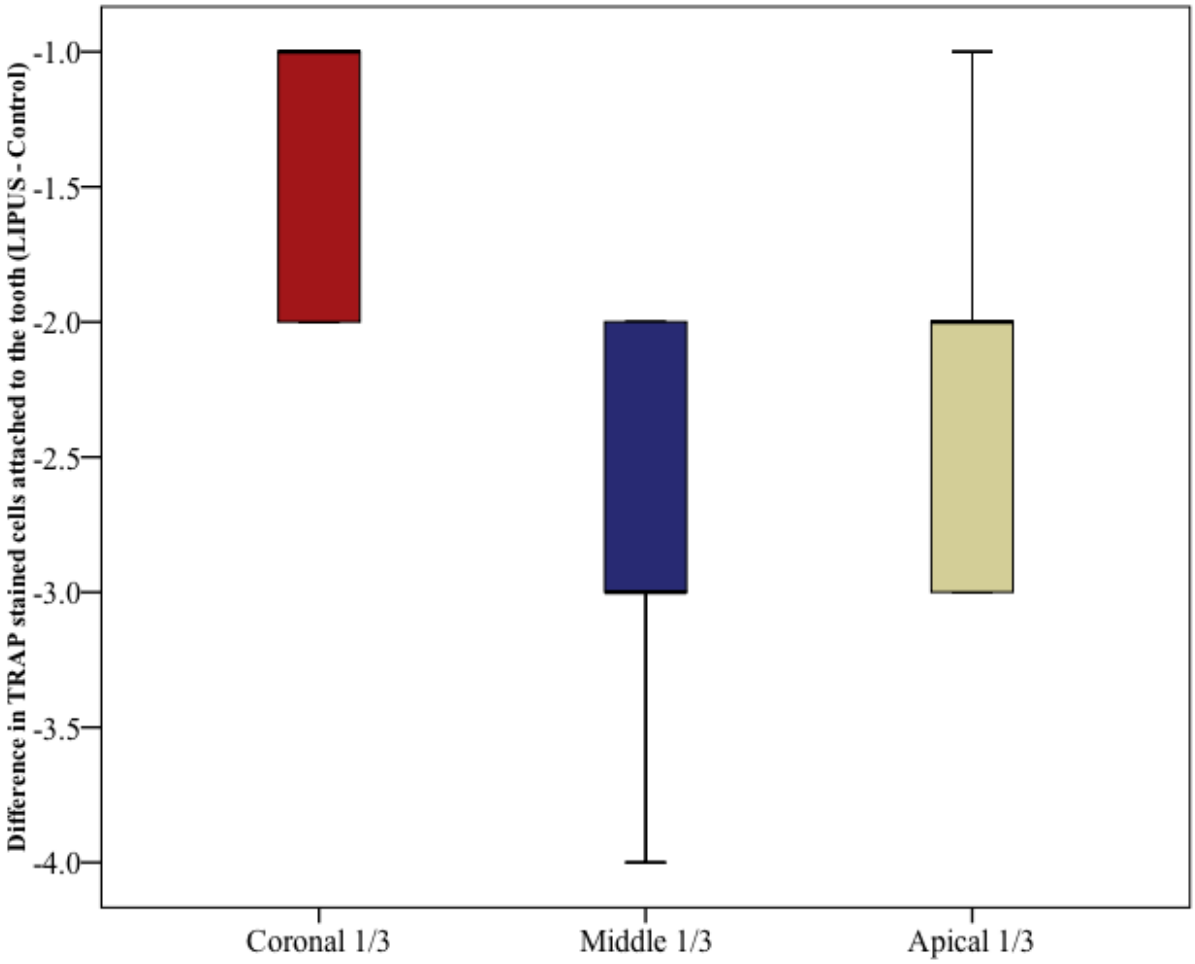

Figure 6.17: Box plot of the difference in TRAP positive cell count attached to the tooth surface inside the PDL (Cemento/odontoclasts) between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side}, showing that it was lowest at the coronal third of the root in the control and LIPUS teeth. But it is lower in the LIPUS teeth at all levels overall.

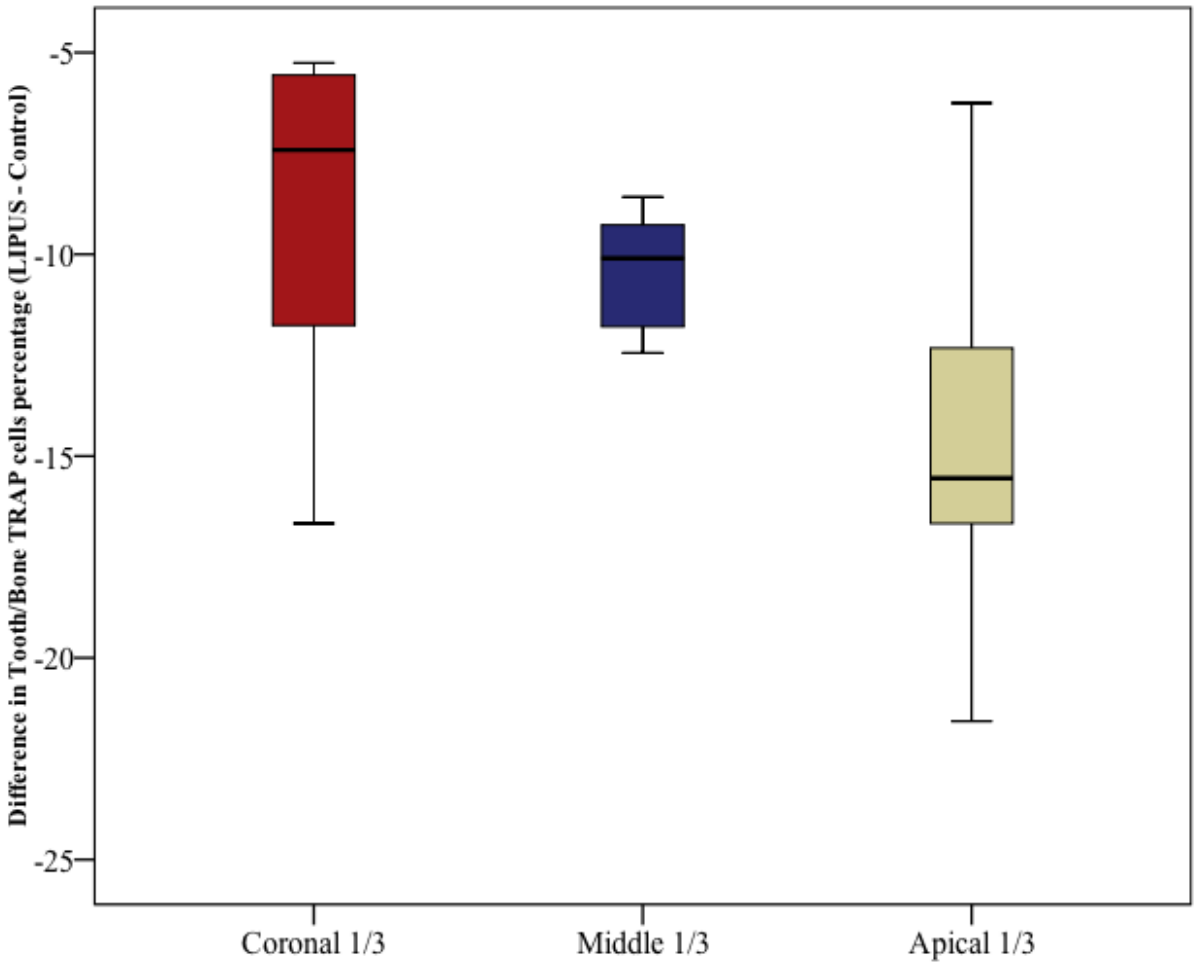

Figure 6.18: Box plot of the difference in percentage of TRAP positive cell count (TRAP positive on tooth X 100 / TRAP positive on bone) between LIPUS and control {The difference variables were calculated as LIPUS side – CTL side}, showing that odontoclasts relative to osteoclast cell count was higher in the control group at the three levels but it was highest at the apical third of the root in the control.

6.4 Discussion:

LIPUS effects were investigated extensively at the *in vitro* level on cell lines and tissue culture models but *in vivo* it was limited to the enhancement of bone healing after bone fractures, osteodistraction or periodontal bone grafting. The interest in LIPUS effects on dentoalveolar tissue response to OIIRR was mainly addressed indirectly through *in vitro* studies and directly with some *in vivo* studies. Despite the large number of studies confirming the anabolic and stimulatory effects of LIPUS on different cell lines and certain clinical situations, the intracellular mechanism is yet unknown and the explanation is limited to mechanical energy stimulation theories. [16,25]

This *in vivo* study investigated the histological findings caused by LIPUS application on orthodontically moved teeth. Histologically we found a lot of similarity between the human and beagle dog periodontium and specifically the cementum layer. Beagle dogs had a gradual increase of the cementum layer from the coronal part of the root toward the apex; also it was similar to humans' by changing from acellular cementum to partially cellular in the middle of the root then being mainly cellular cementum at the apex with cementocytes - cementoblasts trapped in lacunae. LIPUS had a significant stimulatory effect on the lay down of cementum, especially in the middle and the apical thirds of the root in this *in vivo* model. Similar stimulatory effects were found *in vivo* in a periodontal flap surgery model where the LIPUS group had about 70% more cementum deposition than the control while we had about 100% difference [23]. Moreover LIPUS effects on cementoblasts *in vitro* was comprehensively studied and demonstrated stimulatory effects on proliferation, differentiation and excretion of extracellular matrix [17,18,26,27].

Orthodontically induced root resorption has been reported to be inflammatory in nature in response to the hyalinization of the PDL, which resulted from PDL compression and occlusion

of blood vessels [28]. It can start as early as 3 weeks after initiating orthodontic tooth movement [29]. LIPUS demonstrated preventive and reparative potential, where we found less hyalinization in the PDL, precementum layer and reparative cementum in the resorption lacunae. This can be due to LIPUS anti-inflammatory effect [20-22], angiogenesis effect [19,30,31], anabolic effect [17,18,26,27] and anti-necrotic [32] and anti-apoptotic potentials [33]. The close relation of root resorption initiation sites and the areas of hyalinized PDL was observed histologically in this study as documented in previous studies [8], also the close relationship to blood vessels was found.

PDL width was not conclusive of any significant change in the dimension of the PDL due to LIPUS application to orthodontically moved teeth. On the other hand the PDL cellularity was significantly higher. This is consistent with a previous study which reported the stimulatory effect of LIPUS on ligament cell proliferation [34]. Also, the severity of root resorption was shown to be less in the LIPUS group and this was found to be effective in a previous human clinical trial [24]. Although we maintained a continuous orthodontic force applied to the tested teeth with the NiTi open coil springs, the reparative process can still occur with resorption at different areas or at the same resorption lesion [35-37], this was evident and more prevalent in the LIPUS group.

LIPUS was mainly proven to help bone fracture healing and did not have the same effect on intact bone remodelling or density [38]. Unlike dental hard tissue, bone is a highly vascularized tissue and its remodelling lies behind the process of orthodontic tooth movement. We also looked into the pattern of TRAP positive cells to investigate the pattern of odontoclast/osteoclast distribution during orthodontic tooth movement and any changes due to LIPUS application. It was found that the number of osteoclasts was increased due to LIPUS application in the lower two thirds of the root. FEA studies showed that higher stresses at the alveolar crest and lower stresses in the apical region of the PDL [39,40] during bodily and tipping movements, but the

increase in the cementum thickness can increase these stresses [41]. Although in this study we introduced bodily movement and supposedly the stresses are higher at the alveolar crest, the odontoclasts were lowest in the coronal third of the root in both groups. This can be attributed to the large circumference of the root in that area, hence a larger PDL surface area will bear the stresses.

The odontoclasts to osteoclasts percentage was higher in the control group, which indicates that there was more odontoclasts relative to osteoclasts at the investigated areas in the control side compared to the LIPUS side. The percentage was increasing when going toward the root apex, this may be due to the nature of cellular cementum being more prone to resorption because of the lower mineral content and mechanical properties compared to acellular cementum which covers mainly the coronal half of the root. [42,43] Also, this may be due to the fact that the apical third is more prone to resorption as reported in clinical studies [44].

Finally, effects on the dental pulp were in agreement with some *in vitro* studies that investigated the effect of LIPUS on odontoblasts that confirmed an anabolic effect of LIPUS on dental pulp cells. Although the pulp tissue was different from humans in the way that it was highly vascular and didn't have a predentin layer, we still can say the LIPUS effect was stimulatory from the variables we measured and didn't show the harmful effects that were reported in association with some LIPUS intensities *in vitro* [31,45].

In summary, this *in vivo* study proved the preventive action of LIPUS against orthodontically induced root resorption with a potential of increasing the bone remodelling around orthodontically moved teeth. It limited the formation of necrotic PDL due to orthodontic force and occlusion of blood vessels hence the decreased severity of root resorption lacunae. Also, it increased the cellularity and the reparative potential of the PDL in forming reparative

cementum and protective pre-cementum layer. It modified the distribution of clastic cells in the PDL by favoring the resorption of the remodelling bone and reducing the unwanted tooth remodelling. It didn't show any potential for side effects clinically or histologically.

6.5 Conclusion:

LIPUS application simultaneously with orthodontic force presents a preventive effect against root resorption without adverse effects on the bone remodelling around stressed periodontium. Also, it reduced the formation of necrotic PDL and increased the cellularity inside the PDL; it improved the ability of root to repair by laying down cellular cementum and increases the chance of forming protective pre-cementum layer. It also has a stimulatory effect on the proliferation of dental pulp cells.

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Chapter 7:

Discussion and Conclusions

7.1 Thesis summary and discussion:

This thesis addressed the effects of LIPUS on the dentin pulp complex *in vitro* and *in vivo* and its effects on the periodontal tissue *in vivo* during orthodontic tooth movement. In the literature when LIPUS was introduced as a therapeutic mode of treatment it caused different responses in the living tissue. The response was dependent on several factors like the nature of the tissue (cell line, 3-D tissue culture and tissue type), environment (*in vivo* or *in vitro*) and the characteristics of LIPUS (intensity, frequency and application time).

In the application of LIPUS for different periods and different frequencies of application on the tooth slice organ culture in both the long and short term *in vitro* studies, there was an agreement that the application of LIPUS for 20 minutes had a deleterious effect on the tissue and gene expression although other studies reported only initial [1,2] or no adverse effects [3]. On the other hand, the 5 and 10 minutes LIPUS application groups had the most favourable and stimulatory effects on the dentin pulp complex *in vitro* which was well demonstrated with different cell lines [4-7]. The 15 minutes application was stimulatory but to a lesser extent than the 5 and 10 minutes groups; which has been reported to be efficient when applied to cementoblasts cell lines [8]. In the *in vivo* model we only used the 20 minutes LIPUS application that has been tested and well established as an optimal dose and intensity *in vivo* [9-11].

LIPUS demonstrated no significant alteration in the expression of DSPP, TGF β1, RANKL and OPG by the dentin-pulp complex. However, the application of LIPUS for 5 and 10 minutes resulted in a significant increase in the expression of the main structural protein of the dentin collagen type I after harvesting the tissue 24 hours post exposure. Similar effects were previously reported with LIPUS application on cementoblast [12] and odontoblasts cell lines [2]. Also these two groups demonstrated the highest odontoblast cell count and predentin thickness

after 5 days of culture and one session of LIPUS application. In the 10 minutes LIPUS application group, DMP 1 was highly expressed in the short term (24 hours). DMP1 is a major non-collagenous protein that has a regulatory effect on development and differentiation of the odontoblasts [13], the initiation of hydroxyapatite nucleation [14] and regulates the expression of osteocalcin, alkaline phosphatase ALP [15] and controls DSPP which interacts specifically with collagen and initiates hydroxyapatite crystals and controls the rate of crystal growth [16].

From the two long and short term *in vitro* studies we tested the first three hypotheses and answered the questions in regard to LIPUS effect on the dentin-pulp complex. We reject the null hypothesis of the first hypothesis and accept its alternative "LIPUS had a stimulatory effect on the excretion of dentin matrix by odontoblasts and its mineralization process". That was confirmed by the long term findings where predentin layer was increased in thickness. Also by collagen I and DMP1 expression in the short term study. On the other hand, the null hypothesis of the second hypothesis was accepted, LIPUS effect on the excretion ability of odontoblasts was not mediated by the transforming growth factor ß1. Furthermore, we accepted the null hypothesis of the third hypothesis, LIPUS has no effect on the RANKL/OPG based odontoclastic induction mechanism in the human dental pulp whose differentially are responsible for mineralized matrix resorption.

The animal study revealed the effects of LIPUS on the dentoalveolar structures during orthodontic tooth movement *in vivo*. In this split mouth model we found that LIPUS significantly reduced all parameters of orthodontically induced root resorption including the number of resorption lacunae RL by 71 %. This indicates that LIPUS reduced the areas of initiation of the process on the root receiving the treatment which can be attributed to fewer areas of hyalinization in the PDL of the test group and more cellularity in the histological findings, also

the lower number of odontoclasts attached to the root surface from the immunohistochemistry findings. The severity of the resorption process was reduced by 68-70 % due to LIPUS application, which was demonstrated in the values of RL total volume by micro-CT analysis. This was further analyzed and confirmed by the histological findings, which revealed that LIPUS group RL did not extend in the dentin as much as in the control roots. Also the LIPUS group showed a hypomineralized protective [17] layer (pre-cementum) that was not evident on the control roots. Finally, LIPUS demonstrated a reparative effect on OIIRR that contributed to the decrease in the total volume of RL in the micro-CT data, which was confirmed by the histological analysis where we found some RL were filled with reparative cellular cementum.

LIPUS preventive role can be attributed to its anti-inflammatory effect [18-20], which reduced the inflammatory nature of OIIRR. Also, OIIRR inflammatory nature is triggered by the formation of the formation of hyalinized (necrotic) PDL due to blood vessels occlusion. LIPUS had angiogenesis [1,21,22] and anti-necrotic effects [23], which is another possible contributing factors in preventing OIIRR. The anti-apoptotic potential of LIPUS [24] may inhibit OIIRR, because previous reports indicates that anti-apoptotic factors like IL-12 can inhibit both OIIRR and tooth movement [25]. LIPUS anti-apoptotic effect was not reported of being mediated by IL-12 and it didn't adversely affect the orthodontic tooth movement in this study. These effects are antagonist to the nature of OIIRR, which is partially explained by the inflammatory response to the stresses and hyalinization inside the PDL. Hyalinization is a result of PDL compression and occlusion of blood vessels [26].

Cementoblasts are considered to be the main reparative cell line in case of root resorption [27-29]. LIPUS reparative effect can be attributed to the anabolic effect on cementoblasts [6,8,12,30]. These *in vitro* effects demonstrated upregulation of main structural proteins like Collagen-I and

enzymes responsible of mineralization like ALP [6]. Also at the clinical level, LIPUS was reported to minimize root resorption and accelerated healing of the resorption by reparative cementum in humans when used simultaneous with orthodontic tooth movement.[31] In rats, LIPUS was found to regulate the osteoclast differentiation through the OPG/RANKL ratio and initiated reparative effect on orthodontically induced root resorption.[32] These *in vitro* and *in vivo* studies are in agreement with our histological and radiographic findings, which demonstrates the preventive and reparative potential of LIPUS.

Resorption lacunae distribution on the four root surfaces of the tested teeth was not as expected. The buccal surface had the highest number of RL despite that the mesial surface was assumed to be the main pressure side[33-35] according to the applied orthodontic force. The applied force had a mesial vector in order to move the teeth bodily toward the mesial. This can be explained by four reasons; the first is that the fourth premolar was located anatomically buccal to the first molar (anchorage tooth) as seen in Figure (7.1). So the mesially directed force will tend to push the tooth in a mesio-buccal direction. This will result in more compression areas in the buccal PDL than we anticipated. The second reason is that the orthodontic attachment was buccal to the centre of resistance of a double rooted tooth with conical shaped mesial root, around which the tooth rotated bucco-mesially. That may be evident by the orientation of the PDL fibers around the two roots (Figure 7.2). These two reasons could explain the findings from the H & E slides. We found that the buccal PDL was more of a compression area rather than being an area of shear (Figures 7.1 and 7.2). Third possible explanation is that LIPUS had no reparative effect on the root buccal surface because of the amount of LIPUS reached the buccal surface after attenuation being too high and detrimental rather than therapeutic. Also from a two-dimensional FEA done on this fourth premolar, it was estimated that the attenuation ranges from

0-30 % on the buccal and lingual root surfaces. Also the total estimated attenuation when passing through the whole mandible was about 54%[36]. This possibility can be argued because the number of RL was highest on the buccal surface in both the control and LIPUS groups. The last explanation is the fact that most of the buccal bone adjacent to the root is mostly compact bone (Figure 7.2). In previous studies, a strong correlation between OIIRR and root proximity to the buccal/palatal plate of bone was found [37,38]. We believe that the increased RL number on the buccal root surface can be attributed mainly to the proximity of the root to the buccal plate of bone and the tendency of pushing the tooth against this compact bone.

Furthermore, the resorption lacunae distribution can dramatically change if we omitted the data of the two outliers (Animal 6 and 10) from the resorption lacunae count. This would lead to eliminating the dominance of the buccal and mesial surface in the abundance of RL count. This can raise the possibility that root resorption initiation in tension and compression areas of the PDL may not be different, which will contradict with the existing general wisdom of higher root resorption in compression areas. On the other hand, omitting these samples may be argued that they represent 20% of the total sample and can introduce statistical (omitted-variable) bias. Also may be due to the small sample size, these two animals ended up as outliers to the rest of the data.

LIPUS showed differential effect on the odontoclastic and osteoclastic effects as showed in the immunohistochemistry results. We believe this differential LIPUS effect may be due to an indirect effect rather than a direct effect on the osteoclasts and odontoclasts. The main indirect effect would be LIPUS effect on osteoblasts and on cementoblasts are different, because these two types are different [39] although they secret and maintain two biochemically similar and functionally different tissues. [40] Osteoblasts [41] and cementoblasts [42] are known to have an

autocrine-paracrine control on their corresponding clast cells. Also because the source of clast cells are thought to be of hematopoietic origin (monocytes, macrophages) [43], the fact that cementum is avascular tissue while bone is a highly vascular tissue would justify the presence of more precursor cells subjected to the chemotactic factors in the bone more than cementum. Finally, although cementum and bone are similar biochemically, they are very different functionally and structurally [40]. So, because these tissues are the substrate for clast cells attachment and resorptive activity, their actual structural difference may also contribute to the differential effect by LIPUS.

The dental pulp showed no evidence of a pre-dentin layer at the light microscopy level, but LIPUS had a stimulatory effect on it by increasing the cell count but didn't show the harmful effects that were reported in association with some LIPUS intensities *in vitro* [1,2]. Although these findings were different than the results of the TSOC on the long and short term that was expected due to the fact we were testing two different species. Also the response by cell lines, tissue culture models and the same tissue *in vivo* to a certain treatment like LIPUS will differ significantly [44].

Finally, orthodontic tooth movement was slightly enhanced by LIPUS application. The increase was very small and statistically non-significant. Also by taking in consideration the measurement error it can be deemed irrelevant at this point. It should be noted however, that longer study interval with more overall tooth movement might have identified a significantly different rate of tooth movement with LIPUS. From the immmunohistochemistry data, we found an increased number of the TRAP positive cells attached to the bone of the LIPUS treated teeth. The findings support that LIPUS did not affect bone remodelling induced by orthodontic stresses but may have a stimulatory effect. From the previous findings out of the animal study we

accepted the alternative hypothesis of the fourth hypothesis. So can say that LIPUS treatment for 20 minutes per day from the buccal surface during orthodontic bodily tooth movement was effective in preventing and repairing OIIRR *in vivo* without adversely affecting the dentin pulp complex and tooth movement.

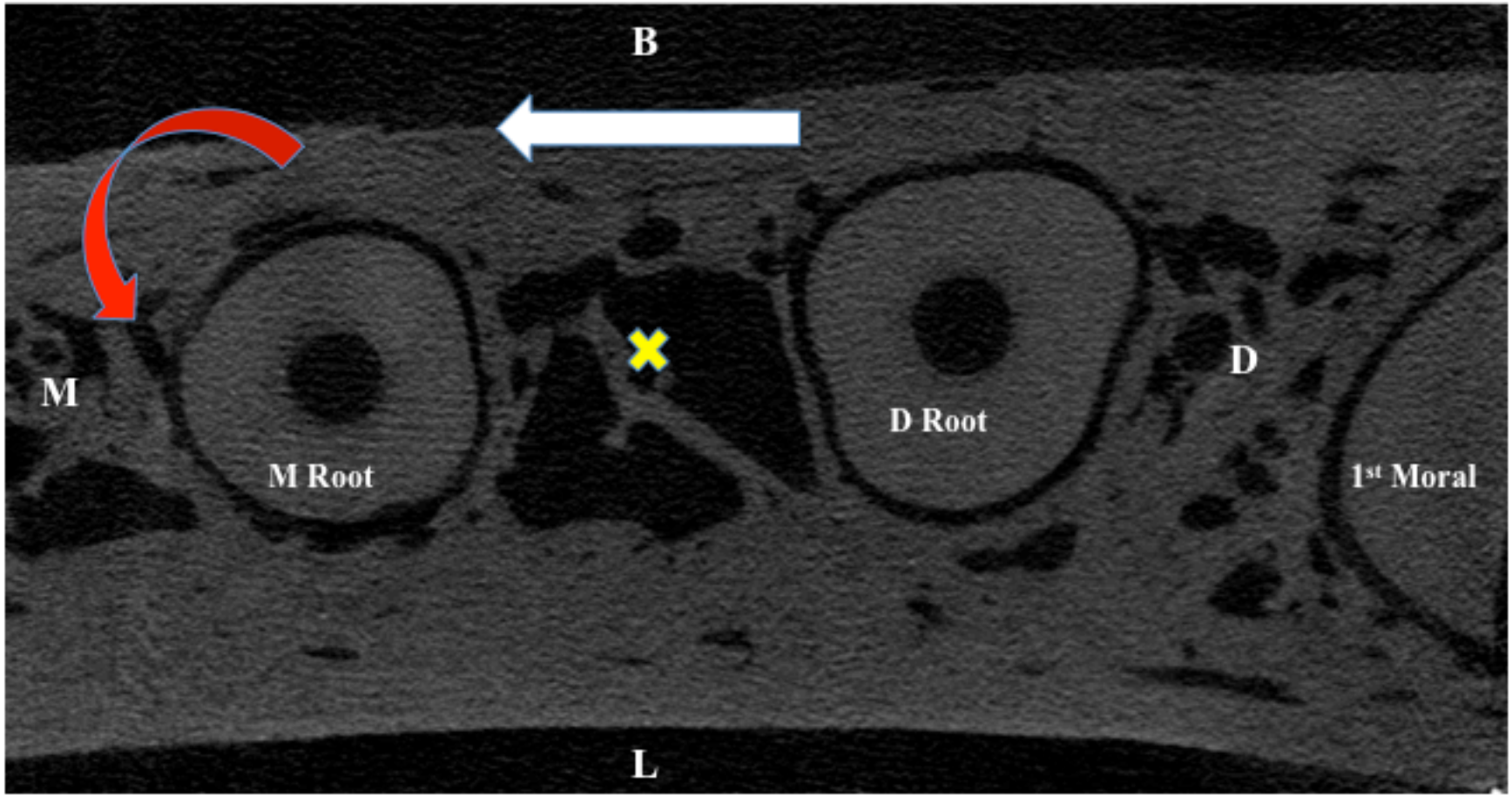

Figure 7.1: Micro-CT image presenting the cross section of the right fourth mandibular molar showing the mesial (M) and distal (D) roots of the premolar. The *white arrow* represents the vector of the applied force and the *yellow X* represents the centre of resistance of the tooth. The *red arrow* represents the direction of the moment produced by the force being applied buccal to the centre of resistance of the tooth. (B: Buccal, M: Mesial, D: Distal and L: Lingual)

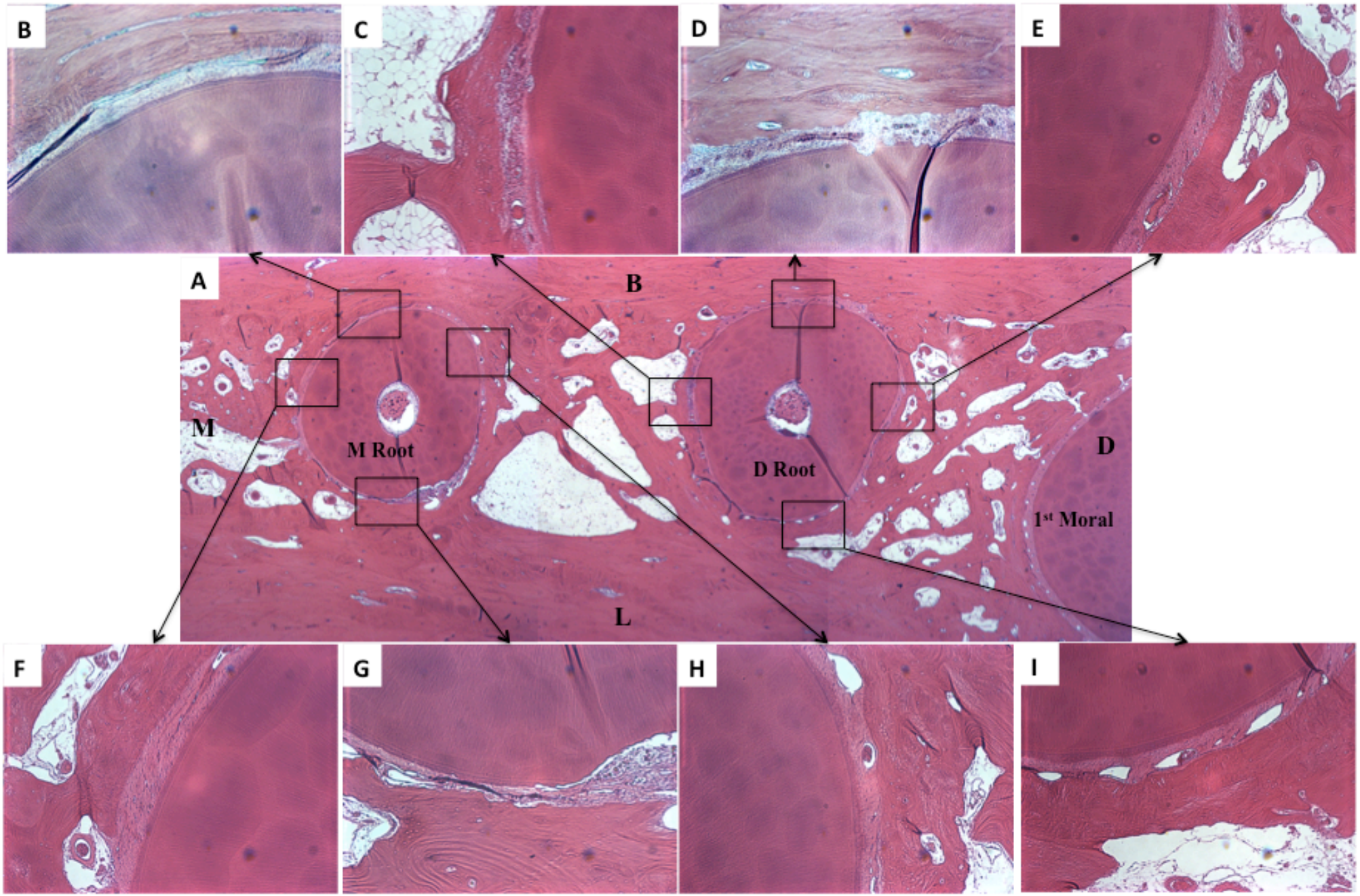
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Figure 7.2: A: Histological picture represents the same micro-CT image in figure 6.19 at a magnification of 1.6X. The orientation of PDL fibers here suggests rotation of the premolar around the mesial root because of the following findings. Instead of mesio-distal tension of the PDL in E and I we can see the PDL fibers are angulated buccally in E and disto-buccally in I. Instead of a shear zone with mesially directed PDL fibers in D, G and B we find these areas are compression zones. F: This area is expected to be a compression site but we can see the PDL fibers are oriented into a bucco-mesial direction representing a zone of shear. C: Compression zone and H: tension zone as expected. (B: Buccal, M: Mesial, D: Distal and L: Lingual)

7.2 Potential Impact

Orthodontically induced inflammatory root resorption is the most common side effect due to orthodontic treatment without a known treatment or preventive protocol due to its multifactorial nature and lack of complete understanding of its underlying mechanism. The process has been attributed mainly to the inflammatory process generated in the PDL due to compressing the ligament and generating a necrotic tissue. Many studies linked the degree of severity of root resorption to several predisposing factors that ranged from the genetic composition of the individual to the design of orthodontic appliance. By introducing LIPUS, we provided a non-invasive procedure that does not require professional expertise, is less expensive than any other solution and is the most conservative among the tested treatment modalities.

From this project we found that TSOC is a very useful and viable option for studying the dentin-pulp complex response to external energy in addition to its usefulness in studying the restorative and chemical materials effects. Also, beagle dogs are an excellent model for studying root resorption and periodontal tissue condition in association with orthodontic tooth movement. Micro-CT imaging is a very accurate and useful tool in quantifying and measuring several variables related to orthodontically induced root resorption with high accuracy at the micrometer measurement unit.

Although the limitations of the Beagle dog model are acknowledged, the present study does provide insight into the potential of LIPUS to prevent and/or treat human orthodontically induced root resorption. The effects were anabolic on

the PDL, cementum and pulp, preventive against and reparative of root resorption at the same time without any adverse effects on the dentin-pulp complex and orthodontic tooth movement. Based on the results of the present animal study, human subject clinical trial research is justified. Although prevalence studies limited severe root resorption to a range from 2-5 % chance [45-47] this number is significant when around 5 million patients started orthodontics in the USA only in 2010 [48].

7.3 Limitations:

The outcomes of this research were conclusive and supportive of he anabolic effect of LIPUS on dental pulp and dentoalveolar tissue with its therapeutic potential on orthodontically induced inflammatory root resorption. Unfortunately some limitations and shortcomings existed in executing and analyzing both models. First, we used different statistical analysis tests in chapters 3 and 4 because of the difference in data meeting the assumptions. But that was mainly due to the difference in sample size and the number of test groups and controls. Second, the tooth slice organ culture was not suitable for extending the experiment for longer than a week of culture due to the changes in the tissue architecture and viability of cells. Also the mRNA isolation was not specific for the odontoblastic cell layer thus we got mRNA from all the pulp tissue cell population. Although that was the case, the tooth slice organ culture is a closer representation of the odontoblastic cell layer *in vitro* than the cell line model [44]. Also the genes we investigated were either: highly specific to odontoblasts like

DMP1 and DSPP [14,16], had a direct effect on odontoblasts function like TGF β1 [49,50] or the main structural components of dentin layer [51]. On the other hand, OPG and RANKL were not specific to the dental pulp and do not express there in normal conditions given that their presence is associated with a resorption process that does not occur in normal pulp tissue [52-54].

In the animal study orthodontic mechanics were executed in with precision to deliver a force with a mesial vector and continuous force at the tube level with a magnitude of 1 Newton of force. Although this appliance was fabricated in an attempt to produce mesial bodily movement of the 4th premolar, the point of attachment was on the buccal surface which is buccal to the centre of resistance of the two rooted premolar and given the shape of the mesial root which was round the applied force produced a moment that rotated the tooth around the mesial root and that was detected by the orientation of the PDL fibers in the H & E stained slides. The results of the H & E histomorphometrical analyses were significant and highly reproducible, but the problem with the hard tissue thickness measurements (cementum and predentin) in both the *in vivo* and *in vitro* studies can be affected by the individual variations and level of sectioning through the root. Vital staining can be the solution for marking the newly deposited hard tissue and minimizing the effect of this error on the final data.

RT-PCR was a very useful method in understanding LIPUS effects at the gene expression level in the *in vitro* model but we could not perform this analysis in the animal model. Although the micro-CT and the histology / immunohistochemistry provided very valuable information, *in situ* hybridization

would have provided us with details at the gene expression level with accurate localization in the periodontal tissue which would explain tissue behavior changes due to LIPUS application. Details about the histology and micro-CT results will be explained at the molecular level if we looked at the RANKL/OPG balance in the PDL to understand the remodelling process. Also looking at the proliferating cell nuclear antigen PCNA [55] and vascular endothelial growth factor VEGF in the PDL and dental pulp will give an insight on the nature of cell proliferation and neovascularization [1,56]. Moreover, looking at the common inflammatory mediators like IL-1, IL-6 and TNF-alpha could assess the inflammatory process [56-58]. Finally, cementum major structural proteins (type I collagen)[59] and glycoproteins (bone sialoprotein and osteopontin); and dentin non-collagenous proteins (DMP1 and DSPP)[60-62] can be viewed in a more dynamic way by this technique.

Last but not least, this trial is very short and simple compared to the actual duration and complexity of orthodontic tooth movement clinically. So, the LIPUS effect on the long term that is more applicable to the clinical environment is still to be understood. Furthermore, patient compliance of applying LIPUS or wearing elastics for regular orthodontic treatment is one of the most difficult challenges orthodontists face with a big portion of their patients, but this can be improved in several ways like the machine design, ease of use and convenience of applying it for the desired time.

7.4 Recommendations for future work:

The plan is to develop a 3-D microstructural finite element analysis (μFEA) model of the LIPUS wave propagation through the biological tissues of a beagle dog mandibular first premolar with the supporting hard and soft tissues and validate this model. Afterward the verified 3-D FEA model will be applied to a beagle dog mandibular fourth premolar and study the pattern of attenuation, the power amount reaching the PDL on each tooth surface and the relationship with the animal study results. This will help in understanding the possible biologic response of LIPUS as it attenuates while propagating through different dentoalveolar structures. Because the amount of ultrasound attenuation during propagation through different biological tissue layers is unknown, the relationship between the intensity of propagated attenuated LIPUS and the biological response of the dental and periodontal tissues has to be analyzed in order to make a decision about power modification and method of application of LIPUS to the region of interest.

In the limitations we addressed the issue of the *in vivo* study being very short compared to real life clinical orthodontics; hence we are planning to conduct animal and human studies that will extend for longer periods of time. The device is already FDA approved but we are also working on a prototype that will be more user friendly and adaptable for different patients' needs. Also we will be working on adjusting the frequency of application times of LIPUS during orthodontic treatment. Although both frontal and undermining bone resorption, which are responsible for orthodontic tooth movement occur within the first two weeks after

orthodontic force application; orthodontic appliances nowadays are designed to maintain low magnitude (optimal) continuous force between activations, which will make it challenging to allocate the time of LIPUS application to a certain time in between activations.

7.4 Conclusions:

From both the *in vivo* and *in vitro* models we tested, we concluded the following findings on the effects of LIPUS:

- Daily application of LIPUS on TSOC for 5 days (long-term) did not have any effect on the predentin thickness layer, and had an adverse effect on the odontoblast cell count.
- A one-time application of LIPUS in a 5-day culture (long-term) of TSOC increased the predentin thickness in all groups. Also it increased the odontoblast cell count in the 5, 10 and 15 minutes application groups but decreased it in the 20 minutes application group.
- Application of LIPUS did not have any effect on the TSOC cell count or predentin thickness in the short-term (24 hours).
- The application of LIPUS for 10 minutes upregulated the expression of collagen-I and DMP 1 in the short term (24 h), where the 5 minutes application upregulated the expression of collagen I only.

- LIPUS did not affect the rate of orthodontic tooth movement and had a trend of increasing it with increased population of the osteoclasts attached to the alveolar bone in the PDL.
- LIPUS significantly reduced the number of orthodontically induced root resorption initiation areas by 71 %.
- LIPUS significantly reduced the total volume of orthodontically induced root resorption lacunae by 68 %, and reduced its volume relative to the affected root total volume by 70%.
- LIPUS induced the formation of precemntum layer, thicker cementum and reparative cellular cementum.
- LIPUS had no adverse effects on the dental pulp *in vivo.*

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Appendix (1)

Animal study raw data of the measured clinical tooth movement and micro-CT variables on both sides (LIPUS-test and control): Number of resorption lacunae (RL), total volume of resorption in μm^3, percentage of root resorption to the total root volume, the RL on each root surface (B: Buccal, M: Mesial, D: Distal and L: Lingual).

	LIPUS								Control							
	Tooth Movement (mm)	RL total #	RL Volume (μm^3)	RL %	RL count/surface				Tooth Movement (mm)	RL total #	RL Volume (μm^3)	RL %	RL count/surface			
					B	M	D	L					B	M	D	L
1	0.54	8	6.8	4.85	3	0	0	5	0.4	36	12.67	8.84	7	11	8	10
2	1.12	8	2.23	1.8	4	0	4	0	0.58	23	14.01	10.82	9	8	3	3
3	0.63	7	2.24	1.3	3	1	1	2	0.5	17	11.99	7.18	5	8	0	4
4	0.8	9	4.56	1.88	3	6	0	0	0.48	22	11.56	5.86	2	13	6	1
5	0.7	6	4.48	2.98	4	2	0	0	0.46	19	5.63	4.06	2	4	8	5
6	0.74	3	1.77	1.04	1	1	0	1	0.56	68	48.88	21.78	22	24	4	18
7	0.65	20	8.84	6.08	6	2	5	7	0.51	33	15.69	10.29	9	3	6	15
8	1	11	8.85	4.88	4	0	4	3	0.55	25	18.11	9.48	8	8	4	5
9	0.9	10	9.57	4.78	4	1	1	4	0.9	37	35.62	15.54	12	12	12	1
10	0.81	8	5.11	2.99	5	0	2	1	1.07	37	23.8	12.43	23	3	4	7

Appendix (2)

Animal study's raw data of measured PDL space in millimeters. The section level represents the area where the micro-CT section was taken where 1 is the most coronal and 5 the most apical.

			Mesial surface/Mesial root					Distal surface/Mesial root					Mesial surface/Distal root					Distal surface/Distal root				
Section Level			1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Animal ID	LIPUS	1	.20	.19	.22	.25	.35	.28	.15	.15	.19	.23	.17	.14	.14	.19	.17	.26	.23	.17	.23	.22
		2	.20	.08	.14	.21	.26	.19	.16	.17	.15	.21	.21	.07	.10	.15	.20	.28	.19	.15	.15	.19
		3	.14	.14	.20	.25	.29	.16	.14	.20	.18	.22	.08	.07	.11	.13	.20	.27	.18	.17	.17	.21
		4	.21	.13	.15	.16	.19	.16	.12	.14	.18	.20	.22	.16	.10	.14	.17	.21	.15	.18	.22	.22
		5	.14	.16	.19	.22	.21	.14	.12	.16	.22	.22	.10	.12	.14	.15	.16	.19	.15	.16	.19	.22
		6	.14	.12	.18	.24	.24	.15	.12	.12	.13	.18	.17	.13	.12	.11	.19	.22	.17	.16	.14	.15
		7	.20	.08	.19	.24	.26	.19	.16	.17	.15	.21	.21	.07	.12	.13	.17	.28	.19	.15	.19	.21
		8	.12	.15	.17	.23	.35	.19	.20	.26	.27	.27	.15	.07	.10	.14	.16	.23	.17	.18	.22	.23
		9	.17	.12	.19	.19	.24	.17	.15	.16	.16	.17	.22	.18	.15	.15	.17	.24	.19	.18	.18	.22
		10	.24	.23	.24	.24	.32	.22	.18	.12	.15	.18	.18	.17	.17	.18	.23	.22	.20	.18	.20	.19
	Control	1	.25	.22	.25	.27	.28	.14	.12	.14	.19	.21	.16	.15	.15	.18	.21	.28	.18	.15	.19	.20
		2	.14	.14	.15	.22	.25	.12	.12	.14	.15	.21	.13	.11	.09	.12	.14	.20	.15	.13	.14	.19
		3	.18	.14	.15	.21	.21	.12	.14	.15	.21	.26	.13	.16	.16	.13	.16	.21	.16	.17	.20	.20
		4	.34	.26	.23	.22	.29	.23	.20	.21	.15	.20	.21	.19	.16	.17	.22	.22	.25	.25	.22	.24
		5	.24	.27	.20	.19	.24	.19	.17	.14	.15	.16	.22	.22	.17	.17	.18	.28	.21	.21	.18	.19
		6	.11	.13	.20	.28	.27	.15	.20	.15	.16	.22	.17	.10	.13	.18	.25	.21	.15	.16	.19	.18
		7	.13	.15	.18	.25	.25	.13	.12	.16	.22	.22	.11	.11	.12	.14	.22	.17	.15	.13	.18	.22
		8	.20	.21	.21	.26	.28	.16	.13	.19	.25	.21	.22	.19	.19	.23	.32	.23	.17	.18	.24	.25
		9	.19	.20	.20	.23	.27	.18	.16	.15	.15	.21	.22	.18	.13	.16	.17	.23	.20	.18	.19	.19
		10	.25	.27	.29	.26	.29	.15	.16	.22	.20	.28	.15	.15	.16	.22	.26	.20	.16	.18	.20	.28

Appendix (3)

Animal study's histology raw data of measured cementum thickness in micrometers (μm). The thicknesses were measured at the three levels of the root (Coronal, middle and apical) on all root surfaces (B: Buccal, M: Mesial, D: Distal and L: Lingual)

		Distal root												Mesial root											
		Coronal 1/3				Middle 1/3				Apical 1/3				Coronal 1/3				Middle 1/3				Apical 1/3			
		B	D	L	M	B	D	L	M	B	D	L	M	B	D	L	M	B	D	L	M	B	D	L	M
LIPUS	1	8.08	25.39	15.84	15.72	177.02	51.11	97.67	217.19	112.53	136.89	46.81	135.37	18.17	26.52	27.16	28.05	189.95	79.34	127.54	159.23	121.89	133.86	141.61	155.79
	2	10.35	15.97	33.74	19.67	89.58	55.12	123.41	212.00	204.43	209.13	227.07	248.99	17.91	21.74	35.77	23.95	181.91	151.81	169.95	145.33	135.33	132.33	162.23	137.86
	3	10.00	19.50	13.56	10.23	45.91	67.35	55.47	169.59	269.80	217.50	210.63	217.13	14.23	14.58	14.63	14.18	155.08	143.96	177.27	102.90	127.22	135.26	152.40	150.43
	4	16.35	15.76	14.88	13.79	111.51	118.61	121.64	130.53	103.23	124.55	125.29	145.30	49.77	43.91	28.67	36.96	71.49	101.05	81.37	73.33	181.11	141.99	105.49	100.51
	5	12.66	27.86	24.09	26.29	131.66	178.56	171.69	157.46	346.79	247.41	264.19	246.08	22.07	21.95	32.90	32.49	123.34	125.54	141.59	128.08	250.39	210.25	216.11	262.13
	6	7.45	30.37	19.02	14.91	163.77	58.44	93.42	205.41	111.13	135.16	38.53	136.31	20.16	23.79	26.69	26.49	176.06	94.77	127.15	170.61	120.02	137.38	137.14	167.56
	7	10.81	21.08	26.20	19.01	82.93	49.77	140.04	205.30	236.11	238.06	204.43	209.13	19.84	22.21	25.52	23.64	169.68	155.60	179.98	174.51	124.13	128.59	136.29	158.98
	8	12.57	14.77	11.52	15.01	36.76	67.10	48.88	172.35	237.50	206.98	208.04	216.87	15.05	13.59	17.84	17.71	142.61	134.97	191.28	187.01	136.12	172.88	178.65	177.88
	9	14.69	13.77	15.11	16.23	111.71	117.12	119.37	120.08	56.91	123.15	131.33	142.05	43.58	48.57	29.95	34.67	65.41	102.28	71.04	68.11	192.93	106.05	153.09	91.24
	10	14.67	26.31	20.52	30.71	134.85	175.48	163.91	167.51	246.08	262.63	254.23	388.91	20.97	22.83	31.14	28.35	130.54	102.94	147.71	127.44	252.23	204.67	225.27	241.22
Control	1	10.28	19.52	16.95	26.86	9.07	28.38	43.30	49.16	86.65	48.19	15.42	53.11	8.67	12.18	14.61	13.84	19.85	33.90	54.66	61.21	90.45	92.34	65.06	79.50
	2	9.47	17.24	23.09	13.79	11.07	27.34	25.56	25.44	124.65	135.78	60.60	127.43	11.06	10.62	15.22	14.73	20.38	23.31	15.05	13.99	49.19	50.67	44.39	43.33
	3	13.51	13.62	10.00	11.29	16.45	29.65	34.36	62.57	106.17	107.76	104.23	102.05	7.14	11.70	6.23	8.73	46.08	64.87	25.64	21.08	26.20	49.77	33.62	40.20
	4	16.11	12.22	11.62	12.41	20.13	30.37	25.74	40.30	31.45	53.97	52.69	58.05	13.61	17.62	12.06	13.31	31.96	24.83	23.62	24.48	41.58	36.38	54.59	72.54
	5	17.69	17.13	13.71	26.38	54.24	68.75	43.98	45.65	56.77	68.74	37.10	39.17	17.14	23.01	19.45	17.32	39.09	40.59	29.31	34.13	28.42	36.33	51.91	59.54
	6	11.72	15.76	13.90	25.05	11.56	22.01	40.44	39.99	76.03	46.08	13.61	43.38	10.61	18.84	16.68	12.29	19.66	29.12	46.42	61.81	48.28	68.39	67.15	44.97
	7	9.77	19.02	22.60	15.37	12.25	25.72	23.32	21.49	129.44	116.23	73.51	121.19	10.37	12.00	14.41	14.71	20.40	22.18	16.92	14.05	44.16	51.17	41.67	47.85
	8	11.53	16.58	12.35	11.04	16.02	23.40	38.74	32.20	115.53	100.59	101.58	96.11	6.49	4.42	9.69	9.89	25.52	80.25	36.63	23.09	27.34	25.56	32.79	44.40
	9	12.94	11.56	12.23	12.67	17.88	32.56	28.73	32.99	26.95	61.18	53.99	60.39	12.55	20.08	14.42	14.94	30.72	27.12	25.96	24.67	41.47	50.29	58.66	68.21
	10	17.30	15.93	12.85	26.66	64.85	72.65	65.01	68.97	57.39	72.75	43.97	47.26	16.43	27.17	16.42	13.18	53.67	50.28	51.77	20.49	29.79	33.70	30.84	60.71

Appendix (4)

Animal study's histology raw data of measured PDL space in micrometers (μm). The thicknesses were measured at the three levels of the root (Coronal, middle and apical) on all root surfaces (B: Buccal, M: Mesial, D: Distal and L: Lingual).

		Distal root												Mesial root											
		Coronal 1/3				Middle 1/3				Apical 1/3				Coronal 1/3				Middle 1/3				Apical 1/3			
		B	D	L	M	B	D	L	M	B	D	L	M	B	D	L	M	B	D	L	M	B	D	L	M
LIPUS	1	110.85	175.73	125.25	111.86	183.31	119.55	132.97	165.57	146.37	129.47	78.71	76.2	163.42	104.98	97.87	108.53	313.32	129.37	142.16	241.51	126.8	83.78	61.68	126.65
	3	145.14	148.07	183.03	119.57	132.53	138.64	103.51	71.86	237.50	206.98	208.04	216.87	145.19	95.94	174.61	95.95	168.98	109.36	128.41	150.84	382.42	350.84	334.30	257.12
	4	212.91	257.61	136.13	35.02	85.63	172.96	89.39	66.73	49.86	119.06	100.71	71.48	129.21	81.67	99.74	82.21	104.05	92.46	74.21	99.21	108.37	87.28	113.55	123.12
	5	67.45	105.39	60.24	49.41	37.46	94.69	61.65	50.08	254.31	286.13	209.25	371.31	51.89	102.57	74.97	49.83	72.50	63.88	72.37	57.95	156.67	163.98	203.46	182.67
	6	127.93	205.05	144.54	124.84	197.98	135.79	148.15	215.74	156.20	149.31	84.08	72.10	166.50	128.77	70.30	104.04	305.24	126.58	151.69	210.91	138.09	105.10	56.07	119.86
	7	103.77	139.21	87.54	18.84	115.77	113.88	223.23	91.81	326.06	302.17	316.39	245.91	79.52	95.07	53.65	38.99	171.25	114.63	110.49	106.82	124.33	153.33	146.47	142.33
	8	132.24	162.08	186.39	100.49	144.08	153.86	105.56	63.42	226.49	243.56	244.51	217.50	149.24	110.30	182.83	96.82	134.16	99.78	106.32	156.68	380.10	364.79	263.26	233.84
	9	184.96	218.88	152.55	49.31	61.34	131.97	101.30	67.66	59.76	114.84	110.16	69.03	116.46	92.52	78.60	80.28	109.67	79.21	58.38	91.89	131.00	94.43	135.58	127.24
	10	80.81	86.89	72.96	43.25	46.19	69.14	84.59	42.54	254.91	257.63	208.33	365.62	57.32	84.62	63.60	41.98	73.71	65.91	61.86	60.78	182.67	182.67	155.17	139.46
Control	1	162.78	163.11	94.27	106.01	183.28	142.71	98.37	105.92	223.00	150.06	111.46	144.89	144.91	74.37	466.08	159.53	115.99	124.28	102.22	117.76	160.18	100.15	152.09	224.48
	2	118.66	133.25	114.07	66.05	89.27	78.66	73.77	39.38	104.97	154.91	120.76	63.65	94.39	87.80	70.35	73.39	90.19	38.47	72.00	79.26	176.52	194.11	289.84	255.26
	3	142.46	149.70	112.47	129.10	120.24	103.62	116.62	122.77	145.41	129.97	217.61	125.99	86.68	62.31	86.62	117.49	163.56	239.28	142.60	130.02	486.96	451.69	395.07	305.82
	4	132.79	135.05	165.62	145.25	136.23	115.57	102.13	106.19	131.30	101.21	98.71	87.93	78.24	66.19	68.80	121.43	120.01	71.24	87.14	136.97	195.99	137.06	93.19	140.26
	5	144.10	167.88	103.43	156.67	106.07	124.99	148.39	168.81	72.67	80.90	53.57	80.13	131.79	67.47	179.72	259.75	84.40	75.94	71.96	63.49	100.75	70.69	116.67	91.03
	6	205.22	163.56	81.80	102.04	186.88	121.04	99.09	95.08	192.75	149.85	102.48	148.36	130.02	83.94	493.01	181.03	128.67	157.14	96.85	122.54	190.78	111.61	188.49	188.24
	7	99.80	147.90	156.23	72.85	81.32	76.82	83.66	37.77	110.98	181.12	138.39	95.29	86.97	81.20	77.05	84.87	77.03	49.95	61.75	74.90	166.28	209.30	253.46	285.79
	8	125.32	151.21	113.05	107.48	104.89	113.61	120.13	139.19	215.62	249.98	284.84	254.96	90.29	73.06	86.96	98.61	162.78	156.12	155.94	163.11	386.67	358.43	333.02	267.13
	9	139.81	127.70	142.97	124.05	144.87	107.58	82.60	128.18	118.94	109.94	100.32	70.57	101.39	70.67	72.03	114.64	124.59	71.86	97.40	147.41	194.42	124.20	169.16	177.55
	10	130.94	197.58	121.33	122.67	91.13	128.97	153.37	177.28	84.80	75.80	62.03	61.09	160.47	86.98	203.70	239.24	74.20	56.77	53.78	64.24	101.25	66.14	115.68	114.86

Appendix (5)

Animal study's histology raw data of measured PDL cell count/1000 μm^2. The cells were counted at the three levels of the root (Coronal, middle and apical) on all root surfaces (B: Buccal, M: Mesial, D: Distal and L: Lingual).

		Distal root												Mesial root											
		Coronal 1/3				Middle 1/3				Apical 1/3				Coronal 1/3				Middle 1/3				Apical 1/3			
		B	D	L	M	B	D	L	M	B	D	L	M	B	D	L	M	B	D	L	M	B	D	L	M
LIPUS	1	7.80	2.70	3.50	5.80	4.70	4.40	4.90	3.70	6.30	3.70	4.70	6.20	9.40	1.90	5.00	3.70	3.00	4.00	4.00	4.20	3.80	5.90	9.00	5.00
	2	5.30	5.40	10.00	7.40	4.50	3.60	7.00	4.30	4.50	3.80	4.50	4.10	6.10	4.90	5.50	5.70	4.30	4.50	7.10	6.20	4.30	5.30	9.00	4.30
	3	5.80	4.10	5.80	4.80	6.00	10.20	7.60	11.10	5.30	5.60	5.20	4.50	6.70	6.60	4.20	7.60	10.00	6.30	7.10	7.00	5.00	6.00	5.50	6.60
	4	5.80	5.40	4.50	9.00	7.60	5.60	8.30	5.80	7.50	6.70	6.30	7.40	7.50	5.60	7.30	3.90	5.60	6.40	6.80	5.00	1.70	5.80	8.90	7.80
	5	4.80	4.30	5.80	7.40	8.20	7.00	7.70	10.60	4.20	4.30	5.80	7.00	6.50	5.80	6.60	7.80	4.60	7.10	8.10	7.30	4.90	6.50	8.00	6.40
	6	8.30	3.00	3.80	5.20	4.50	4.80	4.70	3.80	6.00	3.90	4.40	6.70	9.20	2.00	5.40	3.50	3.20	3.70	4.20	4.90	4.00	5.40	9.50	4.80
	7	4.80	5.90	9.10	8.10	4.70	3.20	7.60	3.80	4.30	4.20	4.30	4.50	6.50	4.30	6.50	4.80	4.70	4.20	7.70	5.60	4.70	5.00	9.40	4.00
	8	6.20	3.80	6.10	5.30	5.60	9.50	8.10	10.10	5.50	5.70	4.30	4.50	6.20	7.30	4.00	8.40	10.80	5.80	7.70	6.30	6.00	5.20	6.10	6.00
	9	6.40	5.00	4.80	9.80	7.10	6.00	7.70	6.60	6.80	7.20	6.00	8.00	6.80	5.00	6.50	4.50	5.00	6.90	6.00	4.60	2.00	5.10	9.00	7.30
	10	5.10	4.60	5.30	7.90	7.40	7.40	8.30	10.00	4.40	4.00	6.20	6.90	7.50	5.30	7.10	7.30	5.00	6.60	8.60	6.70	5.10	6.30	8.60	6.10
Control	1	5.00	3.75	4.00	3.60	3.90	2.10	3.50	4.10	3.20	3.00	4.00	1.70	2.20	3.20	.30	3.80	4.40	2.40	3.30	3.50	3.90	3.80	2.20	2.30
	2	3.80	2.70	2.90	3.90	3.40	2.70	4.20	4.60	4.80	2.20	2.70	2.00	3.00	2.80	2.30	5.30	2.20	3.70	3.00	4.30	1.90	2.30	2.70	2.30
	3	2.90	3.60	2.50	3.70	5.30	4.60	3.30	3.60	2.70	2.90	3.40	2.70	3.80	3.20	2.00	3.50	4.80	3.00	3.40	3.20	5.30	5.60	5.10	5.00
	4	3.30	3.70	2.40	4.90	4.20	2.70	3.60	4.80	3.40	4.20	3.90	4.40	2.50	3.30	4.20	2.60	3.10	3.00	2.90	2.60	1.70	2.80	4.20	4.10
	5	3.90	2.30	4.70	3.10	4.80	3.20	4.20	4.20	2.50	3.20	3.00	3.00	3.80	3.60	3.40	3.60	3.30	3.50	3.80	3.60	2.80	2.90	3.10	3.00
	6	5.30	4.10	4.30	3.30	3.60	1.90	3.20	4.70	3.10	3.20	3.80	1.50	2.00	3.10	.25	3.60	4.70	2.30	3.60	3.10	4.30	3.30	2.50	2.20
	7	3.40	3.10	2.60	3.50	3.70	2.50	4.80	3.60	4.40	2.60	2.20	2.20	3.30	2.60	3.00	4.30	2.60	2.90	3.60	3.70	2.30	2.00	3.20	2.10
	8	3.40	3.30	2.60	4.20	4.80	4.00	3.70	3.10	2.90	3.00	2.80	2.60	3.30	4.00	2.10	4.00	5.00	2.60	3.60	3.00	5.80	5.20	5.60	4.60
	9	3.90	3.30	2.80	5.90	3.80	3.10	3.00	5.30	2.90	4.80	3.30	5.00	2.30	2.80	3.60	2.90	2.80	3.40	2.50	2.40	1.90	2.40	4.90	3.80
	10	4.50	2.60	3.90	3.70	4.00	3.90	5.00	3.50	2.90	3.00	3.40	2.70	4.20	3.00	3.90	4.10	3.70	3.20	4.20	3.30	3.00	2.60	3.40	2.70

Appendix (6)

Animal study's histology raw data of measured odontoblast cell count/1000 μm^2. The cells were counted in the pulp at the three levels of the root (Coronal, middle and apical).

	LIPUS			Control		
	Coronal 1/3	**Middle 1/3**	**Apical 1/3**	**Coronal 1/3**	**Middle 1/3**	**Apical 1/3**
Animal 1	13.70	12.38	7.78	10.31	4.92	5.29
Animal 2	10.13	16.90	7.03	3.86	4.55	7.22
Animal 3	10.35	10.90	5.52	5.52	5.67	5.76
Animal 4	10.64	9.59	5.67	6.56	5.44	6.13
Animal 5	10.84	14.06	7.29	6.52	7.14	6.55
Animal 6	13.75	11.43	7.50	9.09	4.53	5.00
Animal 7	10.97	14.52	7.74	4.08	4.26	7.89
Animal 8	10.18	12.78	6.00	5.20	5.65	4.89
Animal 9	11.18	11.57	5.94	6.42	5.44	5.86
Animal 10	10.26	13.42	7.70	6.80	6.54	6.77

Appendix (7)

Animal study's immunohistochemistry raw data of measured odontoclast, osteoclast cells count and the ratio between them at the three levels of the root (Coronal, middle and apical).

	LIPUS									Control								
	Coronal 1/3			Middle 1/3			Apical 1/3			Coronal 1/3			Middle 1/3			Apical 1/3		
	Osteoclasts OC	Odontoclasts ODC	ODC: OC %	Osteoclasts OC	Odontoclasts ODC	ODC: OC %	Osteoclasts OC	Odontoclasts ODC	ODC: OC %	Osteoclasts OC	Odontoclasts ODC	ODC: OC %	Osteoclasts OC	Odontoclasts ODC	ODC: OC %	Osteoclasts OC	Odontoclasts ODC	ODC: OC %
Animal 1	17.00	0.0	0.0	33.00	1.00	3.03	17.00	1.00	5.88	18.00	1.00	5.56	25.00	3.00	12.00	14.00	3.00	21.43
Animal 2	15.00	0.0	0.0	35.00	2.00	5.71	17.00	2.00	11.76	13.00	1.00	7.69	28.00	4.00	14.29	15.00	5.00	33.33
Animal 3	19.00	0.0	0.0	39.00	0.0	0.0	19.00	0.0	.0	17.00	2.00	11.76	31.00	3.00	9.68	14.00	3.00	21.43
Animal 4	14.00	0.0	0.0	34.00	1.00	2.94	15.00	1.00	6.67	12.00	2.00	16.67	26.00	4.00	15.38	18.00	4.00	22.22
Animal 5	15.00	0.0	0.0	39.00	0.0	0.0	14.00	0.0	0.0	14.00	1.00	7.14	28.00	3.00	10.71	13.00	2.00	15.38
Animal 6	12.00	0.0	0.0	29.00	1.00	3.45	22.00	1.00	4.55	13.00	1.00	7.69	22.00	3.00	13.64	19.00	4.00	21.05
Animal 7	20.00	0.0	0.0	38.00	2.00	5.26	18.00	0.0	0.0	19.00	1.00	5.26	35.00	6.00	17.14	12.00	2.00	16.67
Animal 8	13.00	0.0	0.0	28.00	0.0	0.0	23.00	1.00	4.35	14.00	2.00	14.29	20.00	2.00	10.00	18.00	3.00	16.67
Animal 9	18.00	0.0	0.0	31.00	1.00	3.23	15.00	0.0	0.0	19.00	1.00	5.26	32.00	4.00	12.50	16.00	1.00	6.25
Animal 10	21.00	1.00	4.76	41.00	2.00	4.88	21.00	1.00	4.76	19.00	2.00	10.53	30.00	5.00	16.67	19.00	3.00	15.79