T.R.
UNIVERSITY OF GAZIANTEP
GRADUATE SCHOOL OF HEALTH SCIENCES
FACULTY OF DENTISTRY

THE EFFECT OF HYALURONIC ACID ON THE REMODELLING OF ALVEOLAR BONE DURING ORTHODONTIC TOOTH MOVEMENT

Dler MOURAD
DOCTORAL THESIS

DEPARTMENT OF ORTHODONTIC

THESIS ADVISORS
Assis. Prof. Dr. Merve GÖYMEN
Assoc. Prof. Dr. Fatma Deniz UZUNER

GAZIANTEP
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REPUBLIC OF TURKEY
GAZIANTEP UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES
ORTHODONTIC DEPARTMENT

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Dler MOURAD

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Approval of Graduate School of Health Sciences

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

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This thesis has been read and accepted, in scope and quality, as a Doctor of Philosophy thesis.

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DECLARATION

I hereby declare that this thesis is my own work and effort, had been written by me as the result of my own original research in accordance with academic rules and ethical conduct.

I confirm that where I have consulted the published work of others, this is always clearly attributed. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

This thesis is entirely my own work and I have acknowledged all main sources of help.

20.04.2020

Dler MOURAD

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LIST OF SYMBOLS AND ABBREVIATIONS

1, 25-(OH) 2D3	1,25-Dihydroxyvitamin D3
ALP	Alkaline phosphatase
ATP	Adenosine Triphosphate
BGLAP	Bone gamma-carboxyglutamic acid-containing protein
BMSCs	Bone marrow-derived stem cells
BV/TV	Bone volume/Total volume
COX	Cyclooxygenase
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
GAG	Glycosaminoglycan
H^+	Hydrogen ions
HA	Hyaloronic acid
HE	Haematoxylin-eosin
HIF-1	Hypoxia inducible factor-1
Hz	Hertz
IL	Interleukin
kDa	Kilo Dalton
L-NAME	NG-nitro-L-arginine methyl ester
µA	Micro ampere
M-CSF	Macrophage colony-stimulating factors
MMP	Matrix metalloproteinase
MT	Masson's Trichrome
NO	Nitric Oxide

O_2: CO_2	Oxygen: carbon dioxide
OCN	Osteocalcin
OPG	Osteoprotegerin
OTM	Orthodontic Tooth Movement
PDL	Periodontal ligament
PG	Prostaglandins
PRP	Plasma rich platelets
PTH	Parathyroid hormone
RANK	RANK
RANKL	Receptor activator of nuclear factor kappa-B ligand
RAP	Regional acceleratory phenomena
TxA2	Thromboxane
VEGF	Vascular Endothelial Growth factor

ÖZET

Hyalüronik Asidin Ortodontik Diş Hareketi Esnasında Alveoler Kemik Remodellingi Üzerine Etkisi

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Mayıs 2020, 127 sayfa

Bu araştırmanın amacı; yüksek moleküler ağırlıklı hyaluronik asidin (YMAHA) ve düşük moleküler ağırlıklı hyaluronik asidin (DMAHA) alveoler kemik remodellingine ve ortodontik diş hareket (ODH) hızına etkisini değerlendirmektir. Bu çalışmada 84 Wistar Albino sıcan altı gruba ayrıldı. Grup 1 ve Grup 2 her biri altı hayvandan oluşan beş alt gruba (1.,3.,7.,14. ve 21. gün) ayrıldı (n=6). Grup 3, 4, 5 ve 6 'da bulunan 6 şar adet sıcan ise sadece 21.günde sakrifiye edilerek incelendi. İlk üç grupta çift taraflı üst birinci molarların ortodontik olarak mesializasyonu, nikel titanyum kapalı yaylar kullanılarak sağlandı. Sağ tarafa Grup 1 ve 2'de sırasıyla YMAHA ve DMAHA enjekte edildi, sol tarafa ise her iki grupta da normal salin enjekte edildi. Grup 3'te sadece sağ tarafta salin enjekte edildi. Grup 4 ve 5'te sırasıyla sağ tarafa YMAHA ve DMAHA sadece enjekte edildi. Grup 6 negatif kontrol olarak belirlendi. ODH üç boyutlu dijital modeller üzerinde ölçüldü. Ayrıca , birinci moların intraradiküler alanındaki alveoler kemik hacmi ve osteoklast aktivitesi; histomorfometrik analiz ve nükleer faktör-κB ligandı (RANKL), Osteoprotegerin (OPG), Alkalin fosfataz (ALP) ve Osteokalsin (OCN) immünohistokimyasal tespiti ile değerlendirildi. 14. ve 21.günde Grup 1 ve 2'de deneysel tarafların osteoklast sayısı kontrolden daha yüksek olarak bulundu. 21.günde, YMAHA enjekte edilen gruptaki ODH miktarı, kontrol grubundan 1.4 kat daha fazlaydı. Her iki moleküler ağırlıktaki hyalüronik asitin RANKL/OPG oranını ve ALP seviyesini arttırırken, OCN seviyesini değiştirmediği görüldü. Hyalüronik asit enjeksiyonu, osteoklastik aktiviteyi arttırarak ve alveolar kemik yoğunluğunu azaltarak ortodontik diş hareketini hızlandırabilir.

Anahtar Sözcükler: Alveolar kemik remodelling, Hızlandırılmış ortodontik diş hareketi, Hyaluronik asit, Sıçan, Üç boyutlu model analizi

ABSTRACT

THE EFFECT OF HYALURONIC ACID ON THE REMODELLING OF ALVEOLAR BONE DURING ORTHODONTIC TOOTH MOVEMENT

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Aim of this study is evaluation of high molecular weight hyaluronic acid (HMWHA) and low molecular weight hyaluronic acid (LMWHA) effects on alveolar bone remodelling and orthodontic tooth movement (OTM) velocity. In this study, 84 Wistar Albino rats were divided into six groups. Group 1 and 2 were divided into five subgroups according to time period (1,3,7,14 and 21 days) (n=6). In each of Group 3, 4, 5 and 6, six rats were studied only on day 21. In the first 3 groups orthodontic mesialization of maxillary first molars was achieved by nickel titanium closed coil springs on both sides. In Group 1 and 2, right side was injected with HMWHA and LMWHA respectively, while left side injected by normal saline. Group 3 received saline injection only on right side. Group 4 and 5 received only injection with HMWHA and LMWHA, respectively. Group 6 serves as negative control. OTM was measured on 3-dimensional digital models. Alveolar bone volume in the first molar intraradicular areas were evaluated by histomorphometric and immunohistochemical analysis of receptor activator of nuclear factor-κB ligand (RANKL), Osteoprotegerin (OPG), Alkaline phosphatase (ALP) and Osteocalcin (OCN). On day 14 and 21, osteoclasts number of experimental sides was higher than control in Group 1 and 2. In HMWHA injected group, OTM amount were 1.4 times greater than control On day 21. Both molecular weight of hyaluronic acid increased RANKL/OPG, and ALP level, but didn't altered OCN.Hyaluronic acid injection may accelerate OTM by decreasing alveolar bone density and enhancing osteoclastic activity.

Key words: Alveolar bone remodelling, Accelerated Orthodontic tooth movement, Hyaluronic acid, Rat, Three dimensional model analysis

1. INTRODUCTION

Orthodontic disorders can be classified into dental, skeletal, neuromuscular or combination anomalies, dental anomalies are corrected by orthodontic tooth movement (OTM) from one position to another.

The orthodontic movement is accomplished by application of small magnitude force for long duration so that teeth can be forced to move away from their position in the dental arch to new locations. Long duration of orthodontic treatment, which may be between 1.5 to 3 years, is one of the most important disadvantages. Shorter treatment time is desirable for the patient and the clinician as it decreases the undesired harmful side effects of orthodontic treatment (1).

According to many literatures longer orthodontic treatment is associated with different adverse effects like external apical root resorption (2–4), increased prevalence of dental caries which is found to be higher after 12 month of treatment (5,6) in addition to open gingival embrasure space (7) and poor oral hygiene related problems like periodontitis, gingivitis and development of white spot lesions. Another important side that should be considered is the financial aspect of the long orthodontic treatment, so accelerated treatment is more cost effective for the orthodontist and the patient as well (8). In the light of the previous information most of recent researches in orthodontic are dealing with increasing the rate of OTM. Moving teeth through the dentoalveolar complex is a combination of multiple biological events in which receptor cells act in signalling cascade that lead to bone remodelling and tooth movement (9). The rate of bone resorption and formation, which is called bone remodelling, is the determinant factor of the duration of orthodontic treatment. Bone remodelling process is promoted by bone cells (osteoblasts, osteoclasts and osteocytes), while osteoclasts resorb the bone in the compression side, osteoblasts carry out formation of new bone in the tension side (10).

Acceleration of OTM can be achieved by several ways either by increasing the number of the bone cells involved in alveolar bone remodelling to speed up the rate of bone resorption formation cycle or by decreasing the bone resistance to tooth movement.

The methods of accelerating the OTM can be classified into surgical and non-surgical methods. Surgical approaches include corticotomy, osteotomy, interseptal alveolar surgery and piezocision technique. Surgical technique was approved to be beneficial by several studies in human and animals (11–14), but the invasiveness of surgical operation that is not preferable by orthodontic patients limits the use of surgical methods. Moreover, the acceleration effect obtained by surgical interventions are limited only to the first 2 to 4 months after surgery (15).

One of the most common non surgical methods are device-assisted therapy like direct electric currents, pulsed electromagnetic field, static magnetic field, resonance vibration, and low-level light amplification by stimulated emission of radiation (LASER) which was mostly investigated and gave the most promising results (16–19).

The concept of using physical approaches originated from the idea that applying orthodontic forces to the tooth causes bone bending (bone bending theory) and development of bioelectrical potential on the bended sites. Zengo et al. in his measurements on dog alveolar bone found that the concave site will be negatively charged attracting the osteoblasts and the convex site will be positively charged attracting the osteoclasts (20).

Another nonsurgical methods involves biological approaches that are done by exogenously injecting some molecules responsible for enhancing tooth movements locally or systemically. Examples of these molecules are prostaglandins (PG), parathyroid hormone (PTH), relaxin, 1,25-hydroxyvitamin D (1,25[OH]D), calcium gluconate, interleukin I (IL), receptor activator of nuclear factor kappa-B (RANK) and its ligand (RANKL) (21–33).

Hyaloronic acid (HA) is an unusual polysaccharide that has a simple chemical structure but unique properties. HA consists of a large, negatively charged polysaccharide that participates in defining the properties of pericellular matrices and in transferring signals for proliferating and migrating cells (34).

It is a naturally occurring substance in the human body, found mainly in the joints, the placenta, the eye and the dermis. In fact, roughly 50% of the hyarlonic acid in human body is found in the skin (35).

Zhao et al. demonstrated the effect of HA on the proliferation and osteogenic differentiation of rabbit bone marrow-derived stem cells in their in-vitro study. They reported that the osteogenic process was related to the molecular weight and concentration of HA, so high molecular weight HA decreased the cell adhesion rate in a concentration dependent manner (36).

This genuine substance was approved by many researchers to be effective in accelerating the healing process after extraction by enhancing the revascularization, osteoinduction, osteoconduction and remodelling (37–40).

It appears to be a strong bone-healing material in critical size defects in bone induces a nearly complete healing within no longer than 2 weeks by encouraging osteoblastic cell adhesion and binding of alkaline earth cations such as calcium (41). According to many researches molecular weight of HA play an important role in its efficacy (36,42,43).

Since there are no studies in the literature regarding the effect of HA on orthodontic tooth movement, our study will be the first in its field.

The aim of this study is to reduce the duration of treatment by accelerating alveolar bone remodeling in rats after local injection of HA with two different molecular weight, which will be beneficial for the patient by decreasing the side effect of long treatment and economically advantageous by reducing the chair time and the number of the appointments.

2. LITERATURE REVIEW

2.1 The Concept of Orthodontic Tooth Movement

Teeth continue to move and migrate through out the life span of human being; they pass through different stages from eruption to migration inside the oral cavity into various positions (44).

In general, tooth movement can be divided into three types: eruption, drifting that in some way resemble eruption and orthodontic tooth movement OTM, which combine both pathological and physiological response to external mechanical force. The periodontium play the major role in OTM unlike eruption in which dental follicle is considered the key role (45)

Eruption and drifting are physiological tooth movement that occur slowly and take place in the cancellous bone or in the cortical bone with the direction of growth (46), while OTM is characterized by slow or rapid movement of the tooth depending on the magnitude and duration of the force.

During eruption, teeth changes their position in conjunction to growth and development of the dental and skeletal structure surrounding the dental follicle (44). After they emerge into the oral cavity, various physiological forces are applied by different muscles like lip, cheeks and tongue in addition to masticatory force of the opposite teeth, which lead the tooth to its final position in the dental arch. All these forces working in balance to keep the teeth in the desired position (47).

Any variation in this balancing system will cause the teeth to move to undesirable positions, which may affect the function and aesthetic.

Application of external force to the teeth may alter the biological and biochemical-signalling cascade, and that leads to histological changes in the surrounding tissue.

The periodontal ligament (PDL) cells play the major role in OTM by transferring the mechanical forces into biochemical signals that in turns promote alveolar bone remodeling (45).

Burstone in 1962 suggested three phases of tooth movement. They are:

(1) Initial phase: Occurs immediately after application of force due to the displacement of the tooth within the PDL and it last between 24 hours to 2 days.

(2) Lag phase: This phase lasts from 20 to 30 days in which there a little or no movement at all because of formation of necrosis tissue around the tooth.

(3) Post lag phase: Slow or rapid tooth movement occur in this phase which normally starts after 30 to 40 days (10).

2.2 Biology of Orthodontic Tooth Movement

Orthodontic tooth movement is an association of alveolar bone re-adjustment in response to the mechanical force with reversible injury to the periodontium (45). This tooth movement combines alveolar bone resorption in direction of force application and formation of new bone in the opposite direction. Literatures proposed two main theories for OTM to happen:

1. The alveolar bone bending theory, which combines two main ideas for tooth movement, the mechanical bending of the bone and piezoelectricity of the bone.
2. The pressure tension theory, which also include two explanations for OTM, the hypoxia and the fluid flow.

The first theory that started to explain OTM proposed by Farrar in 1888 and stated that application of external force to the tooth and surrounding tissues results in bending of bone tooth and solid structures lead to tooth movement (48).

In 1969, Baumrind stated that bone is found to be more elastic than other tissues and bended more easily in response to external force, while the bone is held in deformed position, this process is accelerated. This theory help researchers to understand facts such as rapid movement of tooth into extraction space and the relative faster rapidity of OTM in children, who have less calcified and more flexible bone (49).

Basset and Becker in 1962 demonstrated that bending of bone causes two types of electrical effects; electropositive on the convex side that favor the differentiation of

osteoclasts and bone resorption and electronegative on the concave side that showed elevated osteoblastic activity and bone formation (50).

The histological researches by Sandstedt 1904, Oppenheim 1911, Schwarz 1932, hypothesized that a tooth moves in the periodontal space by creating a pressure and tension side (51).

The classical pressure-tension theory suggested that the changes in the chemical environment after application of force stimuli lead to cellular differentiation inside the PDL, which in turn lead to tooth movement. According to pressure tension theory, after 2 to 3 seconds of force loading, the tooth moves within the PDL space that ranges from 0.15 to 0.38 mm, resulting in PDL compression in the side of force direction and PDL stretch in the opposite side. In the compression side the tiny blood vessels of PDL are constricted and blood flow decreased while in the tension side blood vessel are dilated and blood flow is increased, this in turn lead to shifting in O_2: CO_2 level and releasing biologically active agents such as prostaglandins and cytokines (e.g. interleukins). These agents promote differentiation of bone forming cell called osteoblast in the tension side and bone resorbing cell called osteoclast in compression side (51).

Application of heavy orthodontic force leads to abandon blood supply resulting in cell death and formation an area of dead cells around the tooth in a phenomena called as hyalinization. As a result, no osteoclast differentiation occurs within the compressed PDL space; instead, a delayed recruitment of osteoclasts from adjacent bone marrow space is responsible for the “undermining resorption” that removes the lamina dura next to the compressed PDL (51).

Therefore, it usually takes 7 to14 days for tooth movement to occur when heavy force is applied. By contrast, light force only reduces blood flow, allowing quick recruitment of osteoclasts either locally within the PDL or via blood flow. These osteoclasts remove the lamina dura in the process of frontal resorption. Tooth movement begins soon thereafter, usually within 2 days after light force application. Clinically, it is almost impossible to avoid blood vessel occlusion completely, thus hyalinization always occurs to a certain degree and tooth movement is a result of combined undermining and frontal resorption (51).

As defense mechanism of body is trying to return the normal blood supply and oxygen

level to the tissues around the tooth, hypoxia inducible factor-1 will activates vascular endothelial growth factor and RANKL expression in PDL fibroblasts, osteoblasts and osteoclasts in order to increase resorption of the bone in the compression side and release the constricted blood vessels (52).

In addition to the hypothesis of hypoxia another explanation of OTM is loading-induced fluid flow. The fluid flow hypothesis suggested that redistribution of fluid inside the PDL activate a signaling pathway of bone cells.

After application of orthodontic force, the change in the extracellular fluid pressure disturb integrins which are the transmembrane proteins facilitating cell-extracellular matrix (ECM) adhesion. Stimulation of integrins causes release of intracellular molecular responsible for osteoblast and osteoblasts differentiation and ultimately bone and PDL remodelling (53).

2.3 Cells Involved in Orthodontic Tooth Movement

After application of orthodontic force a series of events take place inside the periodontium and alveolar bone at a cellular level.

2.3.1 Osteoblasts:

Osteoblast or bone forming cells are a type of the bone cells responsible for formation of osteoid which is composed of Type I collagen and non collagenous glycoproteins like osteocalcin (OCN) and osteopontin in addition to the secretion of alkaline phosphatase (ALP) that promote mineralization. Osteoblasts contains a complex Golgi apparatus, nuclei and cytoplasmic processes (54). In addition to the main function of osteoblast in formation of bone they have also a role the regulation of bone resorption by interaction of RANKL with receptor on the pre-osteoclasts called RANK-inducing differentiation of osteoclasts.

Osteoblasts secrete also a soluble receptor called as osteoprotegerin (OPG) that binds to RANKL and block the activation of pre-osteoclasts. Therefore, the balance between RANKL and OPG determines the formation and activity of osteoclasts. Resorption is much faster than formation, an area of bone can be resorbed in 2-3 weeks but it takes at least three months to rebuild it (55).

2.3.2 Osteoclasts

Osteoclasts or bone resorbing cells are giant multinucleated cells whose primary function is calcium haemostasis by bone resorption, typically found against the bone surface in depression called Howships lacunae. To enhance the resorption activity of osteoclasts they contains large number of lysozyme, mitochondria and extensive golgi complexes, the borders of the cell are ruffled to increase surface contact area with bone and facilitate the bone resorption (54).

Osteoclasts dissolve inorganic matrix of the bone by proton pump that secrete H^+ ions into the extracellular matrix and release hydrolytic enzymes like collagenase for degradation of organic matrix of the bone, after that small vesicles at the border of the cell transfer the degraded components into ECM then into the circulation (56).

2.3.3 Osteocyte

Osteocyte or the cell of bone matrix is satellite like cells consisting of a cell body with high nucleus to cytoplasm ratio located between bone matrix lamella in areas called lacunae and cytoplasmic process extend into canaliculi channels in the mineralized matrix, the osteocyte cells contacts with each others by gap junctions between cytoplasmic processes (54). Osteocytes has been derived from osteoblasts that occupy osteoids and passively mineralized (57).

The main function of osteocyte is to hold the bone matrix and work as mechanosensory receptors, in addition to secretion of multiple agents promoting bone remodelling process and mineral metabolism (58).

2.3.4 Fibroblast

Fibroblasts are the main cells that synthetize connective tissue fibers like collagen and elastics or reticular fibers, they are elongated spindle shape with tapering processes and one nucleus (54). Formation and differentiation rate of fibroblasts, which also have the potential to differentiate into osteoblasts or cementoblasts, affect the rate of collagen, cement and bone formation (59). During OTM fibroblasts perform collagen production and destruction simultaneously leading to balanced remodelling an adaptation of periodontal ligament (60).

2.4 Alveolar Bone Remodelling

Remodelling is a balanced phenomena characterized by constant resorption and formation of the bone tissue, it is responsible of replacing about 10% to 15% of total bone mass per year under normal conditions (61).

Different types of bone cells like osteocytes, osteoclasts and osteoblasts promote the remodelling process. The activation of bone cells is promoted by mechanical forces, apoptosis, hormones, cytokines and local factors. Osteocytes play an important role in the transformation of mechanical stress into biochemical signals (62).

Cytoplasmic processes or dendrites projected from the cell body of osteocytes are used for connecting these bone cells to each other and to cells of the bone surface. After application of orthodontic force, a displacement of fluid in the canaliculi lead to locally triggered strain at the cell membrane and activation of the affected osteocytes (63). Osteocytes may also send signals for activation of the bone resorption through expression of RANKL and secretion of macrophage colony-stimulating factors (64). Gene expression analysis suggested that osteoclasts activation was done indirectly by osteocytes through modulation of RANKL in osteoblasts. In addition, humeral factors produced and released by osteocytes and secreted through canaliculi into the bone marrow may regulate the differentiation and activity of osteoclasts (65). It has been reported by previous studies that monocytes are differentiated to osteoclasts in the presence of RANKL and macrophage colony-stimulating factors (66). It is therefore sensible to conclude that osteocytes act as leader mechanosensors in bone.

2.5 Acceleration of Orthodontic Tooth Movement

According to a systematic review conducted by Tsichlaki et al. the overall duration of orthodontic treatment with fixed appliance lasts less than 2 years (67). Multiple factors affects the duration of treatment including number of extracted teeth to relieve the crowding, the severity of the case, presence of impacted teeth, need for orthognathic surgery, patient cooperation and the experience of the care provider (1).

Many studies had been conducted for decreasing the duration of orthodontic treatment by different methods, which could be classified into surgical methods and non-surgical methods (68).

2.5.1 Surgical methods

Surgical procedures for accelerating OTM can be broadly classified into osteotomy and corticotomy. Osteotomy is surgical cut in all bone layer including cortical and trabecular bone while corticotomy is incision, cuts or perforations that performed only in the cortical level of the bone (69).

The first attempts to reorganize irregular teeth by osteotomy assisted orthodontic treatment was introduced by Cunningham in 1893 in which he made bone cuts in the mesial and distal interseptal area with luxation of palatally positioned maxillary incisors in order to bring them into correct position and then fixed teeth with wire for 35 days. It was found that this procedure reduced the treatment time to about one third of the actual duration (70). After that, in 1931, Bichlmayr used corticotomy to treat a patient over 16 years old with diastema and protrusion of maxillary incisors after extraction of premolars (71).

In 1959, Köle introduced a case in which cuts in the alveolar bone 1 cm sub-apically as a bone block was made to decrease the physical resistance to tooth movement in open bite patient (72), the invasiveness of corticotomy procedure according to Köle decrease the popularity of this method. In 1983 Frost et al. showed that regional destructive stimuli could result in accelerated differentiation cell activity in the bone and soft tissues; he termed this cascade of physiologic healing processes as the regional acceleratory phenomena (RAP). So according to Frost phenomena, increasing the rate of tooth movement is not due to the decreasing bone resistance only but also the effects of healing process on the rapidity of bone cell activation (73). Wilcko et al. reported rapid tooth movement at a rate of 3-4 times greater than conventional orthodontic treatment with labial and palatal corticotomies (74,75). Many surgical procedures have been performed to invoke RAP with a minimal invasiveness like corticision, piezocision, micro-osteoperforation and discision. The duration of RAP phenomena lasts for about 4-6 months, which give the orthodontist a time for accelerating treatment during this period (76).

Corticision: Corticision or called cortical bone incision is a flapless procedure performed with scalpel used as a thin chisel to separate the interproximal cortices, many studies showed that coritsition accelerated remodelling process in the direction of tooth movement (77,78).

Piezocision: The piezocision procedure is a technique performed by small vertical cuts in the gingiva and periosteum to the cortex of the bone with bone graft placed in the tunnels (79,80). A modification was made to this procedure and given the term piezopuncture in which only perforations were made in the gingiva and cortex of moving tooth. Another modification was done by using piezoelectric knife to make cuts in the bone instead of surgical burs with minimal damage to the bone, it was first introduced by Vercellotti and Podesta (81).

Discision: Discision is a procedure involves the use of disc saw with micromotor to make grooves in the bone adjacent to moved tooth (82). Distraction osteogenesis is another surgical way for accelerating OTM being progressively distracting healing bone after complete osteotomy of bone around the moved tooth. Liou et al. was the first who used custom made distractor for rapidly moving of canine into the extraction space, he showed that canine can be distracted distally about 6.5 mm in 3 weeks but because of its aggressiveness and possibility of ankylosis, more studies should be conducted (11). The most important disadvantages of surgical methods are the aggressiveness of the procedure for the patient, the possibility of harmful effects to paradental tissues and the short duration about 4 months of RAP phenomena.

2.5.2 Physical methods

2.5.2.1 Resonance vibration

Resonance vibration is based on the idea that every object in universe has its natural frequency so if any external object is made to vibrate at the same frequency of the given object then the external object is said to be in resonance with the object under study (61).

Vibration therapy was used in many medical fields such as improving muscle mass in impaired physical mobility patient and elderly persons (83). It was successfully used in enhancing the bone density in patients with musculoskeletal disorders (84) and decreasing the risk of bone fracture more effectively than walking (85).

In dentistry, resonance vibration was used to decrease pain of dental origin, application of 100 Hz vibration to various points of the tooth resulted in reduction in pain intensity of 75 to 100% (86).

Pain from the orthodontic adjustment was found to decreased effectively after application of vibration via powered tooth brush (87,88). It was hypothesized that vibration therapy might have an effects on bone remodelling and OTM because to its role in stimulation of bone.

Researchers investigated different methods of vibration including magnetic, mechanical and ultrasound vibration. Nishimura et al. demonstrated that implementation of additional vibrational (60 Hz, 1.0 m/s^2) for 8 minutes weekly at 3 time points after initiation of OTM in first molars of rats resulted in accelerating tooth movement via enhancing the RANKL expression in alveolar bone without side effect like root resorption (16).

Darendeliler et al. suggested in his study that vibration induced by pulsed electromagnetic field increased the rate of the tooth movement induced by close coils or by magnetic placed between molars and incisors of rats (89).

Previous literatures on the effect of vibration on orthodontic tooth movement and the paradental tissues have so far been limited to animal studies, but following theses studies a new vibrational device designed especially for orthodontic patient had reached the market.

The device was designed by Dr. Jeremy Mao based on his previous researches at the University of Illinois-Chicago. The device was called Acceledent® (OrthoAccel Technologies, Inc., Houston, Texas) and it applies precisely calibrated micro pulse about 25 g with frequency of 30 Hz that transmit through root of the teeth to the surrounding alveolar bone.

An earlier study at the University of Texas Health Science Center - Houston was conducted as a non-controlled clinical trial with a prototype version of Acceledent®. fourteen patients who were undergoing orthodontic treatment with fixed appliance were included in the study and used the device for 10 minutes twice daily, the total rate of OTM was increased significantly about 70% compared to previous literatures and the patient compliance with the device was about 67% (90).

Based on the previous pilot study, Pavlin et al. conducted a clinical randomized controlled trial on 45 patient with maxillary first premolar extraction and maximum

maxillary posterior anchorage divided into two groups Acceledent group for 20 min daily and control group. The results showed that application of low-level cyclic during orthodontic treatment increased the rate of tooth movement about 1.5 folds more than control group (91).

In spite of the effective role of vibration on OTM acceleration, there are still some disadvantages like the necessity of patient cooperation and long term using of the device, in addition to the high cost of the device.

2.5.2.2 Electrical currents

It was demonstrated that application of direct current ranges between one and 100 microampere (μA) to femoral shaft bone of rabbits would cause catabolic activity at anode and anabolic activity at the cathode (92). Davidovitch et al. reported in their animal study that teeth treated by force and electricity for 7 and 14 days respectively moved faster than those treated only by orthodontic force (93).

A split mouth study by Hashimoto et al. on 28 cats divided into 4 groups for 1, 4, 7, 14 days were done by application of 60 g of force to distalize maxillary canine while left canine receive 10 μA of electrical currents, the results indicated faster tooth movement with higher rate of alveolar bone remodelling on electricity applied site (94).

The first attempt to determine the effect of micro electricity on OTM in human was conducted by Kim et al. in which 7 female orthodontic patients who needed canine distalization received 20 μA for 5 hours daily by electrical device fixed to the maxilla. The study reported about 30% higher rate of OTM on the experimental side in comparison to the control side (95).

2.5.2.3 Magnetic field

Previous studies suggested that pulsed electromagnetic field affects the activity of intracellular cyclic adenosine monophosphate and cyclic guanosine monophosphate by increasing the cell membrane permeability for calcium, sodium and potassium (96). According to theses findings considerable interest had been raised to the effect of magnetic field on alveolar bone remodelling.

Darendeliler et al. evaluated the effects of magnetic field on OTM induced in 18 young male guinea pigs, the force was produced by placing a metal spring between incisors in

two groups and samarium-cobalt magnets in the third group and application of electromagnetic field on the second and third groups by especially designed electrical device produced a pulsed electromagnetic field with the frequency of 15 Hz, a positive duration of 200 μs, a magnetic field of 1.8 mT, and a positive rate of 9 T/second, after 10 days of the beginning of the experiment, the results showed that OTM in electromagnetic field groups was greater by about 2.5 folds than control group (97).

Tengku et al. incorporated Magneforce appliance with close coil spring to move the molar of rats mesially and found that application of static magnetic field to OTM by close coils was not significantly effective in acceleration of tooth movement (98).

Showkatbakhsh et al. transferred that effect of electromagnetic field on OTM to new era by a study on human, 10 patients need extraction of two premolars and distalization of canine were included in the split mouth study. To apply the pulsed electromagnetic field on humans, a removable appliance contains a circuit producing 0.5 mT, 1 Hz of pulsed electromagnetic field was used. The appliance produced magnetic field near to the experimental canine 8 hours per day. Distalization of canine tooth was done by close coil spring, and to protect the control canine from the electromagnetic filed produced by the embedded circuits, an aluminum foil was placed in the midline, the study revealed a significant acceleration in canine movement on the test side (99).

According to the previously mentioned studies, static magnetic fields is not effective in acceleration of OTM, while producing electromagnetic field is significantly effective. Still more studies are required to understand the mechanism and compare it with the effects produced by electrical current alone, in addition the need of custom made device and cooperation of patient restrict the use of the methods in acceleration of OTM.

2.5.3 Photomodulation methods

Photobiomodulation means using of low level laser therapy (LLLT) or light emitting diode (LED) on tissue to achieve biological effects.

2.5.3.1 Low-level laser irradiation therapy

It was known that photostimulation increases mitochondrial metabolism, wound healing, regeneration in skin, muscle and nervous tissue (100–103). Based on its effectiveness on different tissue in the body, many studies were conducted to evaluate the effect of LLLT on the bone remodelling process during OTM (104–112).

The effects of Gallium-aluminium-arsenide diode laser on the regeneration of bone in midpalatal suture after maxillary expansion in rats was assessed and revealed a significant increase in bone formation (104).

Kawasaki et al. found in his study that orthodontic force associated with application of diode laser resulted in 1.3 folds higher rate of tooth movement with more anabolic activity on tension side and increased catabolic activity on compression side (105). These results were in conduction to the results of other studies done on rabbits (106).

Most of the studies demonstrated that LLLT increased the velocity of OTM, but very little information was known about the mechanism. Therefore, more detailed histological studies on the cellular level were performed. Fujita et al. examined the effects of laser irradiation on the RANK/RANKL/OPG cascade and found that RANK and RANKL expression was increased significantly in the irradiation group while OPG show no changes. So LLLT may increased the velocity of OTM by induction of RANK and RANKL expression (18).

Another histological study revealed that LLLT increased the matrix metalloproteinase, cathepsin K, alpha (v) beta (3) integrins, fibroblast growth factor expression which are essential for osteoclastogenesis (107,108).

Altan et al. investigated the effects of 820 nm diode lasers with two different doses 1717.2 J/cm^2 versus 477 J/cm^2 on OTM. The results revealed that higher energy dose was more effective in acceleration of OTM and RANKL expression than the other dose, so the acceleration of OTM induced by LLLT was found to be energy dose dependet (109).

After that, multiple studies were conducted to investigate the effect of different types of diode laser on OTM in human and all of them revealed acceleration of tooth movement after irradiation with diode laser of various wave length, output power and power density (110–112).

Cruz et al. aimed to study the effects of diode laser (780 nm, 10 seconds duration, 20 mW, 5 J/cm2) on canine retraction after extraction o premolar. They found that diode laser irradiation for 10 seconds once a month increased the velocity of canine retraction (113).

We can conclude that LLLT increased the velocity of OTM effectively in different doses, but more studies are necessary to establish the efficient dose with the least frequency of radiation.

2.5.3.2 Light emitting diode therapy

Light emitting diode device differed from laser device in many aspects:

1. The output of LED is divergent and the light is diffused and incoherent, while the output of laser diode is converged and the light is concentrated and coherent
2. The LED device emits light by spontaneous emission, while LASER emit light by stimulated emission
3. The emitted light power by LED is lower than LASER
4. The LASER devices need higher current density than LED to work
5. The fiber type used in LED is multimode only, while single mode and multimode could be used in LASER
6. Economically, the cost of LED devices is cheap in comparison to laser devices
7. Because laser is penetrating tissue in higher percentage, laser is more effective but still have more side effects on tissue than LED
8. The LED is considered more safe to the eye than LASER (114)

The infrared light increase the activity of enzyme located in the mitochondria called cytochrome oxidase C which mediates the synthesis of adenosine triphosphate in cells that in turn hasten the cell metabolism leading to increase remodelling and accelerate tooth movement (115). In addition, photobiostimulation increase the proliferation of cardiac stem cells and mesenchymal stem cells in vitro, which in turn enhance the vascular activity leading to more rapid bone remodelling (116).

Based on previous facts, multiple studies had been conducted to investigate the effects of LED on OTM. Kau et al. measured the rate of change in OTM during levelling and alignment phase of treatment in 90 patients (73 in experiment group and 17 control group), extra oral OrthoPulse® device (Biolux Research, Vancouver,BC, Canada) which produced infrared light of 850 nm wave length was used in experiment groups for 20 min/day, 30 min/day, 60 min/week, respectively. It was observed that crowding resolution was faster in all experimental groups in comparison with control group that only received orthodontic appliance without radiation (115).

In the same year an animal study conducted by Ekizer et al. used OsseoPulse LED device that produce light of 618 nm wave length to investigate the rate of OTM changes. Using of OsseoPulse daily for 10 days resulted in accelerating orthodontic tooth movement with less root resorption (117).

The photomodulation therapy could be considered as effective method for acceleration of OTM, with lower range of side effects on the root of the teeth, Goymen et al. evaluated the effect of two types of photomodulation therapy on root resorption of premolar teeth after application of force. The study implied that no significant root resorption was observed in premolar root after application of LED or diode laser to moved teeth (118).

2.5.4 Chemical methods

Systemic or local administrations of certain chemicals have been found to be effective in accelerating tooth movement.

2.5.4.1 Prostaglandins

Prostaglandins are a group of lipid compounds derived from arachidonic acid by the enzyme cyclooxygenase and naturally produced as a paracrine hormone acting only near to the site of secretion (119), in addition to various physiological tasks in the body PGs had an important role in bone resorption process (120–122). They are almost synthetized in all body tissues as a response to physical, chemical, mechanical, immunological and hormonal stimulation. Yamasaki et al. showed increased level of PGs during OTM in a study conducted on laboratory rats (123). Another study by Davidovitch et al. on cats also demonstrated a high level of endogenous PGs associated with OTM (124). Exogenous systemic or local administration of PGs had been proved to be effective in accelerating OTM in animals, local injection of PGE2 results in enhancement of OTM with no significant differences between single injection and multiple injections that repeated weekly (21).

Oral administration of PGE1 analogue (misoprostol) was effective in doses between 10 to 100.0 μg/kg (125). Another study concluded that local deliveries of PGE1 and PGE2 to the buccal mucosa of monkeys can approximately double the rate of OTM. PGI2 and thromboxane TxA2 also increased the number of differentiated osteoclasts and resorption rate of the bone (126).

Histological comparisons between local and systemic administration of PGE1 was demonstrated by Lee et al. and revealed more osteoclasts number with the systemic route of administration (127). Most of the studies on the relation of PGs to OTM was conducted on animals because the most important disadvantage of PGs is increasing the pain after administration due to release of histamine, bradykinin, serotonin, acetylcholine and substance P, from nerve endings both peripherally and centrally (128).

Yamasaki et al. first conducted experiments on human after addition of lidocaine with PGE1. The tooth movement rate was increased about 1.6 on the injection side (22). Seifi et al. introduced combination of calcium gluconate with PGs to decrease the harmful effect of PGs on root resorption. The combined injection of calcium gluconate with PGE2 reduced the OTM but still in higher rate than control group. In addition, root resorption in this group was the same as control group in spite of the higher rate of root resorption associated with PGs injection without calcium gluconate (31).

2.5.4.2 Parathyroid hormone

Parathyroid hormone is excreted from four tiny parathyroid glands situated in the neck behind the thyroid glands. It is the key factor in regulation of calcium level in blood stream by its effect on kidney (decrease the secretion of calcium by kidney), bone (breakdown to release calcium) and intestine (increasing the absorption of calcium from food). Elevated level of PTH increased the bone remodelling process resulted in either bone formation or bone resorption depending on the form and duration of elevation (129).

Due to its effect on bone resorption, it takes the attention of researchers as an agent to hasten OTM. In 1969, a study demonstrated more shifting of the left maxillary incisor of six rats from midline after local injection of PTH in the distal aspect in comparison to the control group which received normal saline injection (130). After that, another study conducted in 1981 by Midgett et al. was conducted to investigate the effect of hyperthyroidism on OTM in beagle dogs. After initiating hyperparathyroidism in experimental group by feeding the dogs a diet deficient in calcium and phosphorus for ten weeks, this study revealed more OTM in test group than in control group (131).

Some et al. showed that continuous systemic delivery of 10mg/100g of body weight/day of PTH (1-84) is the most effective dose in which the rate of OTM was increased about 50% in comparison with control group (23).

To exclude the harmful effect of systemic exogenous PTH on the bone tissue, local administration was preferred. PTH mixed with methylcellulose gel, to slow the release of PTH due to the high viscosity, was locally injected in the mesial aspect of rat first molar after force application and revealed higher velocity of OTM in experimental group (24).

The pattern in which PTH is delivered is another important aspect for bone remodelling. Continuous infusion and chronic local injection were more effective for accelerating OTM than intermittent administration even that intermittent administration might be effective way to accelerate OTM (132).

In the light of previous studies, it was found that administration of exogenous PTH increases the velocity of OTM in dose dependent manner and relating to mode of delivery of the PTH, but until now all the experiments had been done on animal models and no study investigate the effect of PTH on tooth movement in human.

2.5.4.3 L-Thyroxine

L-thyroxine is a synthetic hormone identical to thyroxine exerted by thyroid glands and has an important role in the calcium metabolism and enhancing the activity of all cells proliferation. The effect of various patterns of administration on OTM has been investigated.

Systemic administration of exogenous thyroxin by addition of 0.003% to the drinking water of a rat model for 4 weeks revealed increased rate of OTM (133). Intraperitoneal administration of L-Thyroxine in different doses showed that 20 mg/kg per day is an effective dose to accelerate OTM (134). Data acquired from a case report of 11 years old girl with hyperthyroidism undergo orthodontic treatment showed increasing in the tooth movement of impacted canine during hyperthyroidism periods (135).

2.5.4.4 Osteocalcin

Osteocalcin (OC), also known as bone gamma carboxyglutamic acid-containing protein is the seventh abundant protein of bone, it is a non-collagenous protein of the bone and

dentin, which produced by osteoblasts and considered as a marker for bone formation in its intact form and marker of bone resorption in fragments form (136), several studies showed that OC is a chemical attractant for osteoclast cells and osteoclasts progenitor cells (137).

A study by Defranco et al. implied that bone particles deficient of OC are degraded lesser than normal bone particles when embedded subcutaneously into rats (138). Another study revealed that pre-treatment of hydroxyapatite particles with OC hasten the appearance of osteoclasts cells (139).

Due to its ability to enhance osteoclasts to resorb bone, it is possible to have an effect on OTM. Local administration of OC in different doses revealed that 1 μg of daily injection into the palatal bifurcation of the first molar in rats results in increasing the rate of OTM (140,141).

Until now, all the researches of OC relation to OTM had been done on animal models, more specific studies should be done to investigate the mechanism of OC effect on OTM in human.

2.5.4.5 1,25-Hydroxyvitamin D

Vitamin D or what is called sunshine vitamin is a steroid hormone and fat-soluble vitamin synthetized from cholesterol when the skin is exposed to the sun. It has many functions in the body; furthermore, it affects various cells responsible for bone remodelling and promotes the absorption of calcium and phosphorus from intestine. Vitamin D is found in the diet in two main form; vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Multiple actions occur in the body to convert vitamin into active form 1,25-dihydroxycholecalciferol (142). This active form of vitamin D is one of the most known stimulator of bone resorption. In spite of its half-life in the plasma is only from 2 to 3 hours, it still activate osteoclast for several days (143). The effect of 1,25[OH] D on bone resorption gave an idea of possibility of its relation to OTM.

Collins et al. found in an experiment done on cats that weekly intraligamentous injections of a solution of 1,25[OH] D3 in dimethyl sulfoxide increase the rate of OTM by 60% more than teeth in the control group (29).

Another study to investigate the most effective dose of 1,25[OH] D3 found that daily injections of 20 μL of 10^{-10} mol/L for three days stimulated osteoclast and increased their number to three fold than control group with saline injection (144). A comparison

between PGE_2 and 1,25[OH] D3 effect on OTM was revealed that 1,25[OH] D3 was more effective in promoting bone remodelling by maintaining a good balance between bone resorption and bone formation (28). The need of repeated injection of vitamin D to reach higher rate of OTM decrease the clinical practicality of vitamin D as an accelerating agent.

2.5.4.6 Epidermal growth factor

Epidermal growth factor (EGF) is a protein consisting of 53 amino acids involved in dermal and epidermal regeneration through binding to receptors on cells promoting migration and proliferation of keratinocyte, endothelial cells, and fibroblast (145).

It was found in some literatures that EGF has an anabolic effect on periosteal bone and increase the catabolic activity for endosteal bone in growing mouse (146), so it logically may have relation to OTM and alveolar bone remodelling. A study by Saddi et al. demonstrated higher rate of OTM after injection of EGF alone or combined with liposome into the root furcation of first molars in rats (147).

Another study conducted by Alves et al. showed that administration of 20 ng of EGF with liposome increased the OTM and osteoclastogenesis significantly compared to the control group which receive only phosphate buffer saline solution (148).

Because the source of EGF in rodents is from submaxillary glands, effects of EGF on bone remodelling and OTM was investigated by sialoadenectomy of submaxillary glands, the results of this study revealed that the decrease in EGF in the experimental group was associated with decrease in OTM compared with the control group (149).

2.5.4.7 Plasma rich platelets

Plasma rich platelets (PRP) is defined as autologous concentration of platelets in small volume of plasma and is known to be rich of autologous growth factors which are key factors in multiple cell proliferation events (150). It was found by an in-vitro study that the PRP affected alveolar bone remodelling in different patterns depending on concentration, high concentration of PRP decreased the proliferation of alveolar bone cells while low concentration stimulate the viability and proliferation of bone cells (151).

Another in vitro study of the effect of PRP of different concentrations on osteoblasts and fibroblasts revealed that concentration of 2.5 times the concentration in whole blood was the most effective in the cells proliferation and higher dose results in suboptimal effect on osteoblasts and fibroblasts functions (152). Based on the previous studies, it was obvious that PRP might have an effect on OTM. Gulec et al. investigated the possible effects of PRP prepared in two concentrations (5 times and 2 times) the concentration in whole blood on OTM in rats in split mouth design. Constant force of 40 cN were applied using close coil between incisor and first molar and the injection of 10 μL of the two concentrations was done at the beginning of the experiment and not repeated again, the amount of tooth movement was measured by superimposition of 3 dimensional (3D) digital models on day 3, 7, 14, 21, 60, the result of this study showed that high concentration PRP increased the OTM 1.7 folds more than the control group and 1.4 folds greater than the moderate concentration PRP (153).

Another study by Rashid et al. in mongrel dogs revealed that local injection of PRP in combination with calcium chloride increased the rate of OTM by approximately two fold in comparison to the control side with higher resorptive activity in the experiment side especially significant in the third week of experiment which lasts for 9 weeks (154). Sufarnap et al. found in their study on guinea pigs that no significant difference between PRP and control group presents but the tooth movement in day 12 was still increased in the PRP groups, while the OTM in the control groups was already stabilized (155).

After achieving effective results from animal studies without macroscopic and clinical side effects, recent studies went toward investigation of the effect of PRP on OTM in human. Liou et al. developed a regime for injection in human as one injection for levelling and alignment at the beginning of treatment and another injection after six months for retraction of anterior teeth or protraction of posterior teeth (156).

2.5.4.8 Corticosteroids

Also called steroids are a group of substances used as anti-inflammatory drugs for wide range of conditions; its name was derived from an internal hormone produced by the adrenal cortex. Cortisone is the most famous type of this group that used in treatment of many inflammatory and autoimmune diseases such as rheumatoid arthritis, skin diseases, ulcerative colitis, adrenal insufficiency and asthma (157). The effects of

corticosteroid on the bone turnover was demonstrated in different studies but the mechanism by which corticosteroids supress bone formation and increase bone resorption is still not really understood (158). The relation between corticosteroids induced osteoporosis and OTM was investigated by Ashcraft et al. in a study included 16 New Zealand rabbits divided into four groups two of them are control groups and two of them received 15 mg/kg of cortisone acetate for 4 days before the application of orthodontic force to induce osteoporosis; this study revealed more rapid OTM and subsequent relapse in treatment group than control group (159).

To elucidate the effects of corticosteroids on OTM more clearly, a study was conducted on 64 rats divided into three groups; a chronic group received 8 mg/kg/day of methylprednisolone injection subcutaneously for 7 weeks with OTM for the last 3 weeks; an acute group injected by the same amount of methylprednisolone for 3 weeks associated with orthodontic force application; and a control group without injections but only OTM. The chronic use of corticosteroids resulted in more significant acceleration of OTM than the acute and the control group (160).

While previous studies demonstrated acceleration of OTM after prednisolone injection, oral delivery of 1 mg/kg of prednisolone in rats for 12 days induction periods followed by 12 days of OTM showed no significant difference in the velocity of OTM, which may be related to short duration of steroid administration (161).

Recent study by Abtahi et al. used 16 rabbits divided into two groups experimental group that injected by triamcinolone acetonide 1mg/kg/day intramuscularly for 21 days and control group with no drug injection, both the groups simultaneously had orthodontic appliance bonded to upper incisors for 3 weeks, significant increase in OTM by 2 folds was observed in the experiment group compared to control group associated with less osteoblasts number in steroid injected group and less resorptive lacunae in control group (162).

Due to the systematic side effects associated with corticosteroids administration, it is not considered as an effective acceleration method but still further researches are needed to clearly understand the mechanism of function.

2.5.4.9 Nitric oxide

Nitric oxide (NO) is toxic colourless gas, which plays a number of important roles in the body, including vasodilation of blood vessels, control oxygen supply to the mitochondria, killing parasitic organisms and virus infected cells, it is synthesized from Arginine through the enzyme nitric oxide synthase (163). It also plays an a role in bone turnover and regulation of osteoblast-osteoclasts function (164).

The studies that investigated the relation of NO to OTM applied either NO precursor to increase the amount of it at the site of injection or NO synthase inhibitor that called N(gamma)-nitro-L-arginine methyl ester (L-NAME) to decrease the synthesis of NO.

Shirazi et al. investigated the role of NO in OTM, 48 rats divided into four groups; two sham group with no injection and with saline injection respectively, and two test group with local injections of 200 mg/kg L-arginine (NO precursor) or 10 mg/kg of L-NAME daily for 11 days. OTM was started at the time of the injection and continues 2 days after finishing of injection protocol, it was found that the L-arginine group showed higher rate of OTM and L-NAME group showed lower rate of OTM compared to control groups (165). Hayashi et al. demonstrated the same results of Shirazi et al. study with injecting of L-NAME every 3 days for 21 days in Wistar Albino rats (166).

Erol et al. investigated different concentrations of L-arginine and L-NAME (10^{-4}, 10^{-5} and 10^{-6} mol/L) and the results revealed that 10^{-4} mol/L of L-arginine was more effective in acceleration of OTM than the other while 10^{-4} mol/L of L-NAME significantly decreased OTM more than other groups (167).

2.6 Hyaluronic Acid

Hyaluronic acid is member of glycosaminoglycan (GAG)'s family, which are polysaccharide compounds consist of multiple units of disaccharides. It performs many functions in our body. Because of its high viscosity, it supplies joints with necessary lubrication and enhance water retention in skin in addition to promoting specific cell receptors for proliferation and migration. Hyaluronic acid can be found in high levels in cartilage, heart, intervertebral discs of the spine, skin, connective tissues, umbilical cord, synovial fluid and the vitreous of the eye (168).

Hyaluronic acid is dispersed in different amount throughout the body of human: about 4 g/L was found in synovial fluid of joints, 4g/kg in the umbilical cord, 200 mg /kg in the skin and about 10mg/L in the thoracic lymph with total of about 15 g in 70 kg human body (169). Because most of the HA of human body is concentrated in joints and skin one of most common symptoms of decline in synthesis of HA is joint discomfort and skin wrinkles.

2.6.1 History

In 1880, the French chemist Portes described a new mucin in the vitreous body that acted differently from other mucoids in cornea and cartilage, he named the new mucine (hyalomucine), this was probably the first uncovering of HA. After that, many researchers tried to isolate this new polysaccharide. In 1918 Levene and Lopez-Suarez drew out a material called mucoitin-sulfuric acid from vitreous body and umbilical cord, which was proved later to be HA extracted together with a mixture of sulfated glycosaminoglycan (170). In 1928, Francesc Duran Reynals found an active substance in the testicles of rabbits that caused depolymerization of HA, this specific enzyme was called hyaluronidase (171).
Actually HA, as an isolated material, was discovered by Karl Meyer and John Palmer in 1934 extracted from vitreous of bovine eyes. The name HA was derived from Hyaloid which means glass and uronic acid (172). Over the next 10 years, Meyer and other authors distracted HA from various animal organs; for example, it was found in joint fluid, the umbilical cord and recently it has become possible to extract HA from almost all vertebrate tissues.

Another source of HA from the capsules of *Streptococci* groups A and C was discovered. In 1937 by Kendall et al. which was the most economical and reliable source for the industrial production of HA (173). During the second half of the 20th century, HA was found to have clinical applications, mostly for eye surgery, treatment of joint diseases and aesthetic medicine. In 1943, during the Second World War, Nikolay Gamaleya constructed a complex bandages to treat frostbitten soldiers. The new dressing received the name ‘Regenerator’ and the main component of the bandage was an extract from the umbilical cord which was considered to be one of the most important industrial sources of HA (174).

After the Second World War, application of HA in several medical branches was started. In the 1950s, Endre Balazs investigated the HA effect on the treatment of retinal detachment by prosthesis. In 1969 it was reported that HA was used in order to prevent postoperative soldering. HA was then used for treatment of arthritis and was injected into the joints of racehorses that suffered from arthritis. A few years later, Stegman and Miller started to use HA in implanted intraocular lenses (170). Because of the novel and innovative formula of HA, it has become one of the most important components in ophthalmology and has found extremely wide applications in aesthetic medicine (175). Nowadays, HA-contained products are the 'gold standard' for injectable cosmetics.

2.6.2 Physical and chemical properties of hyaluronic acid

Hyaluronic acid is quite different from other GAG in most of the properties. It is the only not sulphated GAG and the only one that synthetized in the inner face of plasma membrane rather than golgi apparatus.

The biochemical structure of HA was first discovered by Meyer in 1958 (176). It consists of many units of disaccharides; the disaccharide in HA is glucuronic acid and acetylglucosamine linked together into one unit (**Figure 2.1**); it can be up to 25,000 disaccharides long (168).

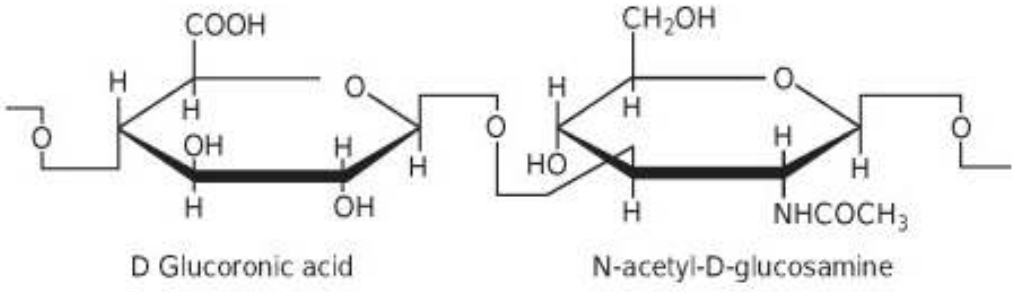

Figure 2.1. Chemical structure of hyaluronic acid (177)

The molecular weight of HA ranges from low molecular weight as 10^4 dalton to high molecular weight 10^6 dalton, while the density of HA is about 30 - 200 kg/m^3 (168). At higher concentrations, solutions have an extremely high viscosity. 1% solution is like jelly, but when it is put under pressure it moves easily and can be administered through a small-bore needle; therefore, it had been called a "pseudo-plastic" material. The extraordinary hydrophilic properties of HA solutions make them ideal substances as lubricants (178). HA is naturally synthesized by a class of integral membrane proteins

called hyaluronan synthases (HAS), of which vertebrates have three types: HAS1, HAS2, and HAS3 (179).

In mammals, there are three enzymes responsible of degradation of HA hyaluronidase, b-d-glucuronidase, and β-N-acetyl-hexosaminidase. These enzymes may be found in the extracellular matrix intracellulary or in the circulation. In general, the function of hyaluronidase is restricted to degradation of high molecular weight HA into smaller oligosaccharides while β-d-glucuronidase and β-N-acetylhexosaminidase further cleave the oligosaccharide fragments (180).

One of unique characteristic of HA is its ability to absorb water in large amount, so it is considered as one of the most hydrophilic (water-loving) substances in nature. HA molecule acts like a sponge and can absorb from 500 to 1000 times its own weight of water. So it can be described as nature's moisturizer (35).

2.6.3 Mechanism of action of hyaluronic acid

Underlying the role of HA in various cell behaviours is the interaction with binding protein called hyaladherins that interact with HA to regulate many aspects of cell functioning such as cell migration, cell-cell adhesion, and cell differentiation. Some of these proteins interacts with HA in extracellular matrix, while others bind to it at the plasma membrane (181). So hyaladherins could be divided into two subgroups matrix hyaladherins and cell surface hyaladherins. Examples of matrix HA binding protein are aggregan, which is found in the cartilage matrix, versican, is widely distributed in nervous tissue and soft connective tissue, neurocan, a chondroitin sulphate proteoglycan of brain. Cell surface hyaladherins had been detected in different tissue and display a competition for binding of HA by HA hexasacharides (182).

2.6.4 Cytotoxicity

The term cytotoxicity is used here to describe events that interfere in cell synthesis, causing cellular dysfunction and structural damage (183). HA is highly resorbable, biocompatible, non-antigenic and non- immunogenic, and non allergic owing to its high structural homology across species, and poor interaction with blood components (184,185). Boeckel et al. investigated the effect of high dose of HA on osteoblast proliferation in vitro with the absence of hyaluronidase enzyme, the results indicated an acceptable cell viability about 72% in the presence of HA (186).

2.6.4.1 Carcinogenicity

It has been shown that the HA level is elevated in various cancer cells and it is believed to form a less dense matrix, thus enhancing the cell's motility as well as invasive ability into other tissues (187,188). It has been shown that the over expression of HA synthases increases the HA level, which leads to the acceleration of tumor growth and metastasis. On the other hand, exogenous oligometric HA inhibits tumor progression, most likely by competing with endogenous polymeric HA (189).

2.6.5 Applications of hyaluronic acid

The unique viscoelastic nature and the biocompatibility of HA make it the key element in many medical products, especially in improving the treatment of joints disorders such as osteoarthritis, temporomandibular joint disorders, cartilage damage, eye diseases like detached retinas and glaucoma, dry eyes, wrinkled skin, prevention of scarring, and wound healing (190–196).

2.6.5.1 Hyaluronic acid in orthopedics

Orthopedic application of products containing HA in different concentrations was approved to be beneficial for the development of cartilage, the maintenance of the synovial fluid and the regeneration of tendons (197). Viscosupplementation of HA in treatment of osteoarthritis help in improving the state of inflamed joint by lubrication and enhance the mobility in addition to in role in reducing pain (198). HA is being administrated into osteoarthritis patients via two main ways either oral administration or local injection, most of the trade products available now are different in:

1. Molecular weight ranging from 500 to 6000 kDa
2. Methods of production either animal source or bacterial bio-fermentation
3. Molecular structure such as linear or cross-linked
4. Method of crosslinking
5. Concentration ranging from 0.8–30 mg/mL
6. Volume of injection 0.5–6.0 mL
7. Posology and dosage

Some of these products are Synvisc, Synvisc-One, Gel-One, Hyalgan, Supartz FX, Orthovisc, Euflexxa, previously named Nuflexxa, Monovisc, and Gel-Syn. Studies had shown that both local injections and oral supplementation of HA can combat

osteoarthritis symptoms, especially those with early osteoarthritis (199).

2.6.5.2 Hyaluronic acid in ophthalmology

Hyaluronic acid occupies an important role in the ophthalmology, it has been used in ophthalmologic surgery as a space-filling matrix in the eye to hold the shape of the anterior chamber and prevent adhesion of intraocular lenses. Furthermore, it serve as a viscosity enhancing component of eye drops and as an adjuvant to eye tissue repair (175).

2.6.5.3 Hyaluronic acid in cardiovascular surgical operation

Application of HA during surgical operation had been restricted only to prevent postoperative adhesions (180). Using of biomaterial surfaces treated with cross-linked HA in cardiovascular operations had been associated with reduced platelet adhesion and thrombus formation (180). Furthermore, sulfated HA derivatives can act as heparin mimics because it possess a negative charge density that allows it to interact and inhibit the coagulation process (200).

2.6.5.4 Hyaluronic acid in dermatology

Hyaluronic acid holds a prestigious position between skin care products and cosmetics. So many famous companies produced thousands of skin care products containing HA in different concentrations linked to other minerals and vitamins or pure HA. Skin care products containing HA were applied in various formulas like serum, cream, gels, powder, emulsions, masks and oral supplements. Several studies were conducted on the effect of dermo cosmetic HA as an antiaging product (201,202).

In addition to its role in moisturizing the skin, it was used as a dermal filler to overcome the age related face wrinkles. Different types of injectable HA fillers are currently available like Belotero Balance, Juvederm Ultra, Restylane, and Voluma. Each of these HA fillers has unique characteristics, which determine location(s) on the face they are best injected, what level they should be injected in the skin, and their performance and duration of effect after injection. For example; Belotero Balance and Restylane Silk are often used for the correction of shallow and fine lines of face, Juvederm Ultra is used more for improving lip volume, most of these products could be used to reduce the appearance of nasolabial folds and marionette lines. Finally, cheek augmentation is most commonly performed with either Voluma to create structure, elevate, and reposition the cheek into a more youthful appearance (203).

The results offered by HA filler might last from few months to over a year depending on the location and performance of the filler. For instance; the lips filler may last 6 months but in nasolabial folds and cheeks it may last more than 9 months to 12 months (204). HA dermal fillers have been used for forehead lines, temples hollowness, superior orbital rim, glabella, lower eye lid bag, malar, cheek hollowness, nasolabial folds, lips, fine lines around the mouth, earlobes, and volume loss on the back of hands (204).

2.6.5.5 Hyaluronic acid in pharmacology

Hyaluronic acid has been investigated as a drug delivery agent for various administration routes, including ophthalmic, nasal, pulmonary, parenteral and topical. It is thought to act as either a mucoadhesive and retain the drug at its site of action/absorption or to modify the in vivo release/absorption rate of the therapeutic agent (205).

2.6.5.6 Hyaluronic acid in oral surgery

Recently, application of HA products in dentistry has been attracts many researchers. The benefit of HA to enhance healing after tooth extraction was investigated by many researchers in animals and humans (38,39).

Several studies investigated the effect of HA on prognosis of dental implant and osseointegration and it revealed that topical application of HA may activate bone formation in the placement of endosseous implants thus it could be able of accelerating osseointegration process (206,207).

2.6.5.7 Hyaluronic acid in periodontology

Multiple products were fabricated for topical use in periodontology and were supplied as intraoral spray, hydrogel, and mouth rinse like *Gengigel* or as injection ready to use during periodontal surgery like *Hyadent.* The topical preparations of HA were proved to be effective in controlling plaque induced gingivitis and gingival bleeding as the topical application of 0.2 % HA gel *Gengigel* in addition to routine oral hygiene maintenance showed a significant clinical improvement in gingival parameters (208).

Another study conducted by Pistorius et al. revealed a beneficial effect of HA containing spray as an adjunct to non surgical periodontal treatment of plaque induced gingivitis (209). Jentsch et al. observed a substantial enhancement of plaque indices,

papillary bleeding indices and gingival crevicular fluid measurements after application of HA gel for 3-weeks (210). The role of HA in the surgical treatment of gingival recession and chronic periodontitis was well documented in previous literatures, application of 0.8 HA gel *Gengigel* appeared to improve the gingival recession value and clinical attachment level in association with modified Widman flap surgery (211).

Loss of inter dental papilla and formation of black triangle is one most manifested aesthetic problem faced daily by orthodontist, periodontist and restorative dentists. HA can be successfully used for construction of missing papilla (212). Singh et al. proposed that 5% HA injection is effective for reconstruction of interdental papilla (213).

2.6.5.8 Hyaluronic acid in orthodontic

Sadikoglu et al. described in his paper the effect of HA on the bone formation after rapid maxillary expansion and concluded that local injection of high molecular weight HA stimulated the bone formation and reduced the risk of relapse after expansion of maxilla in rats (42).

2.7 Histomorphometry

Stereology is the method of calculating the 3D dimensions of an object by measurements on the two dimensional microscopic sections (214). The basic strategy for stereological measurements is the probability sampling methods such as systematic random sampling, which means the that every point of the object has the equal opportunity for selection, this sampling method depends on systematic rule for selection of samples (215).

In the calculations such as area or volume ratios, the most important step is to select the samples randomly in a systematic way at every stage of the procedure, the randomness of samples protect the investigators from bias (216). Calculating the volume of three-dimensional objects can be done depending on the Cavalieri principle in which the unregularly object is divided into systematic parallel slices of known thickness, then calculating the volume of each slice by multiplying the surface area by thickness of the slice. After that, adding theses separate volume together to obtain the total volume of the object (217).

This method is considered highly efficient with coefficient of error about 5%, to increase the reliability of the method, The calculation of surface area could be done by

using the dotted area measurement ruler, however, for obtaining more precise results, some semi-automatic machines or image analysis systems with special software can be used (218).

2.8 Immunohistochemical Markers

2.8.1 RANKL

RANKL is abbreviation of receptor activator of nuclear factor kappa-B ligand and it is a transmembrane protein composed of three identical units of polypeptide known to stimulate osteoclast activity and bone resorption. In addition to bone tissues, it is found in lymph nodes, thymus and lung, and at low levels in a variety of other tissues including spleen and bone marrow (219).

RANKL is produced mainly by osteoblasts cells and activated T cells and bound to Receptor activator of nuclear factor kappa-B (RANK) receptor, which produced on osteoclasts and their progenitors. The interaction between RANK and RANKL is responsible for osteoclast differentiation, formation and activation (220). Determination of RANKL amount in bone tissue by anti-RANKL antibodies is considered as an important value in studies of physiological or pathological bone resorption like OTM or bone diseases.

2.8.2 Osteoprotegerin

Osteoprotegerin (OPG) is member of tissue necrotic factor receptors family, but it is secreted and acts like cytokines. It is considered as a binding partner of RANKL to inhibit differentiation and function of osteoclasts so it is called bone protector because it protects the bone from excessive resorption. It is produced mainly by osteoblasts and osteogenic stromal stem cells, it is found in many tissue other than bone like heart, kidney, liver, spleen and bone marrow (219), the ratio of RANKL/OPG is considered the key factor in osteoclastogenisis and regulation of bone mass (221). Therefore, detection of the level of OPG in a bone tissue is a good indicator of osteoclasts activity and bone remodelling process.

Multiple previous reports revealed that OPG prevented RANKL from binding to its receptor RANK and decrease the number of mature osteoclasts that are capable of bone resorption.(222) (**Figure 2.2**)

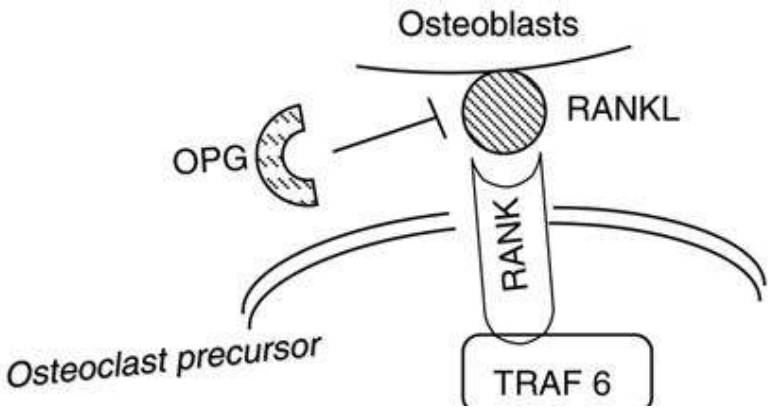

Figure 2.2. The essential signalling pathway for normal osteoclastogenesis under physiologic conditions.

2.8.3 Alkaline phosphatase

Alkaline phosphatase (ALP) is a prevalent membrane glycoprotein that considered as an ectoenzyme attached to the outer surface of osteoblasts in bone tissue. It can be divided into four main types according to the location (placental ALP, Intestinal ALP, germ cell ALP and Liver/Bone /Kidney ALP). ALP plays a profound role in bone metabolism, so it is considered as an important biomarker for bone formation (223,224).

The ability of ALP to hydrolyze phosphate groups at an alkaline pH is known to be associated with formation of bone. Detection of ALP in bone tissue is indicator of osteoblast positive.

2.8.4 Osteocalcin

Osteocalcin (OCN) is a small noncollagenous protein hormone found in bone and dentin, produced exclusively by osteoblasts (225). Recent studies revealed that OCN inhibit bone mineralization (226). Mature osteocalcin is secreted into the bone micro-environment and then undergoes a conformational change that aligns its calcium-binding bone γ-carboxyglutamic acid (GLA) residues with the calcium ions in hydroxyapatite. This property was initially proposed as a mechanism that enables OCN to initiate the formation of hydroxyapatite crystals (227). OCN inhibits the precipitation of calcium salts from saturated solutions (228), so down regulation of OCN results in over mineralization of bone. OCN was proposed as means to bridge the matrix and mineral fractions of bone tissue. Such an arrangement is compatible with the formation of dilatational bands that are seen when bone fractures. In this situation, OCN and osteopontin might serve to prevent crack growth by stretching and dissipating energy (229,230).

3. MATERIAL AND METHODS

The experimental protocol had been reviewed and approved by the Committee of Experiment Animal Care in Gaziantep University (GAUN-HADYEK). (Date: 08/01/2019, Decision no: 2019/3, Protocol no: 82)

Eighty-four male Wistar-Albino rats (277.35 ± 50.16 g, 10 weeks old) were obtained and accommodated in Gaziantep University Laboratory Animal Science Center.

3.1 Materials and Equipment Used in the Experimental Procedure

All materials, laboratory equipment and programs that are used in this study are listed here in sections according to their usage with their manufacturers details.

3.1.1 Materials and equipment used in animal laboratory

- Sterile insulin injector 1 CC (BD Micro FineTM, Istanbul, Turkey)
- Hamilton injector 50 micron Hamilton (Bonaduz AG, Switzerland)
- Straight Mathieu Plier
- Light wire cutter
- Nickel titanium close coils (G&H Orthodontics, Franklin, USA)
- Light cured glass ionomer cement (3M UnitekTM Multi-Cure Glass Ionomer Orthodontic Band Cement, Monrovia, California, USA)
- VALO™ Cordless Led curing light (Ultradent, South Jordan, Utah, USA)
- Round and fissure diamond bur
- Microdrill (Omnidril 35, World Precision Instruments, Sarasota, Florida, USA)
- Rodents mouth retractor (iM3 Inc., Vancouver, USA)
- Dynamometer: force gauge 25-250 g (Correx, Haag-Streit, Berne, Switzerland)
- Polysiloxane impression materials (Oran wash L, Zhemarck, Rovigo, Italy)
- Zeta 7 solution (Zhemarck, Rovigo, Italy)
- Special acrylic impression tray
- Digital weight scale (Precision balance PFB, KERN & SOHN GmbH, Balingen, Germany)
- Digital camera (Sony alpha 6000, Japan)
- İnspection glass (MAX Detail 2X, Eschenbach, Germany)

3.1.2 Chemical substances and pharmacological agents

- 10% Ketamine hydrochloride (Ketasol, Richter Phrama AG, Wels, Austria)
- 2% Xylazine hydrochloride (Basilazin, Bavet, Istanbul, Turkey)
- High molecular weight HA (HMWHA) (Synvisc hylan G-F 20, Sanofi-Aventis, Quebec, Canada)
- Low molecular weight HA (LMWHA) (Hyalrat, Fidia Farmaceutici S.P.A, Padua, Italy)
- Normal saline (NS) 0.9% sodium chloride (Turktipsan, Ankara, Turkey)

3.1.3 Materials used in histological investigation

- Buffered 10% Formaldehyde (Noratex, Istanbul, Turkey)
- Hydrochloric acid/formic acid solutıon (Biodec R, BIO-OPTICA, Milano, Italy)
- Tissue processor (Thermoscientific, ExcelsiorES, USA)
- Xylene (Leica, Wetzlar, Germany)
- Mayer's Hematoxylin and Eosin staining kit (72804E, Microm, Walldorf, Germany)
- Microscopic slide (Patolab, Beijing, China)
- Parafine (Leica, Wetzlar, Germany)
- Parafine embedding workstation (Histostar, Thermoscientific, Massachusetts, USA)
- Microtome blade (Feather, 12031985P, Osaka, Japan)
- Automated Microtome (Leica RM2255, Wetzlar, Germany)
- Light microscope (Leica DM5000 B, Wetzlar, Germany)
- Image analysis program (Leica Qwin.Plus, Cambridge, UK)
- Distilled water
- Pure Alcohol (Ethyl Alcohol) (Sigma-aldrich, Darmstad, Germany)
- Alcohol 96% (Sigma-Aldrich, Darmstad, Germany)
- Aqueous mounting media (AML060, Scytek, Logan, Utah, USA)
- Mouse RANKL antibody (Santa Cruz Biotechnology, California, USA)
- Mouse anti-OPG monoclonal antibody (Santa Cruz biotechnology, California, USA)
- ALPL polyclonal Antibody (Elabscience Biotechnology Inc., Texas, USA)
- Mouse monoclolonal osteocalcin antibody (Santa Cruz biotechnology, California, USA)

- 3,3′-Diaminobenzidine DAB Quanto 4ml Chromogen 125ml Substrate (Richard-Allan Scientific, Michigan, USA)
- Streptavidin-Biotin Amplification kit (Histostain plus, Zymed , San Francisco, USA)

3.1.4 Three dimensional scanning equipment and programs

- 3Shape lab scanner (D-250; 3Shape, Copenhagen, Denmark)
- 3Shape dental system premium Software version 19.1 (3Shape, Copenhagen, Denmark)
- Dental CAD software (Version 2016.10 Valetta, Exocad, Darmstadt, Germany)
- Image J (Bone J2 plugin, version 1.5, Wyne Rasband)

3.2 Care of the Experimental Animals

Eighty-four adult male Wistar Albino rats, 10 weeks old and weighing 281.34 ± 48.4 g, were raised and housed in Research Animal Laboratory of Gaziantep University (GAUNDAM). The rats were kept in polycarbonate boxes at temperatures between 19°C and 22°C, and humidity of 40% to 50%, in a photoperiod of 12/12 hours starting at 8 am.

3.3 Groups and Study Design

Two types of HA were used in the experiment; HMWHA (Synvisc, Sanofi-Aventis, Quebec, Canada), which has a molecular weight of 6000 kDa, and LMWHA (Hyalrat, Fidia Farmaceutici S.P.A, Padua, Italy), which has a molecular weight of 500 to 750 kDa. Split mouth design was used in the study with right side always was considered as the experimental side and left side was considered as the control side.

Total of 84 Wistar Albino rats were divided into six main groups (**Table 3.1**):

Group 1 (GI) consists of 30 rats distributed evenly into five subgroups according to the time of sacrification (GI-1: scarified on day 1, GI-3: scarified on day 3, GI-7: scarified on day 7, GI-14: scarified on day 14, GI-21: scarified on day 21). Every animal in this group received orthodontic appliance on both left and right sides. After that, HMWHA was injected into the right side and normal saline (0.9% NaCl) was injected into the left side.

Group 2 (GII) consists of 30 rats distributed evenly into five subgroups according to the time of sacrification (GII-1: scarified on day 1, GII-3: scarified on day 3, GII-7: scarified day 7, GII-14: scarified on day 14, GII-21: scarified on day 21). Every animal in this group received orthodontic appliance on both left and right sides like GI, but LMWHA was injected into the right side and 0.9% NaCl was injected into the left side.

Group 3 (GIII) consists of 6 animals received orthodontic appliance on both sides and sacrificed after 21 days, but injection was made only on the right side with 0.9% NaCl, while no injection was done on the left side. The purpose of this group was to elucidate of the effect of 0.9% NaCl on OTM.

Group 4 (GIV) consist of 6 animals received only HMWHA injection on the right side, 0.9% NaCl injection on the left side without orthodontic appliance on the right or on the left and sacrificed after 21 days. This group serve as negative control for GI to exclude the effect of HMWHA injection on physiological tooth movement.

Group 5 (GV) consist of 6 animals received only LMWHA injection on the right side and 0.9% NaCl injection on the left side. Orthodontic appliance was not used in this group. This group was sacrificed after 21 days and serve as negative control for GII to exclude the effect of LMWHA injection on physiological tooth movement.

Group 6 (GVI) consists of 6 animals without injection and without orthodontic appliance. This group serve as control group of all study groups and sacrificed after 21 days.

Table 3.1. Groups and study design

Groups	*Number*	*OTM*	*Injection*		*Time points*
			Right	*Left*	
G I	30	Yes	HMWHA	0.9% NaCl	Day 1, 3, 7, 14, 21
G II	30	Yes	LMWHA	0.9% NaCl	Day1, 3, 7, 14, 21
G III	6	Yes	0.9% NaCl	-	Day 21
G IV	6	No	HMWHA	0.9% NaCl	Day 21
G V	6	No	LMWHA	0.9% NaCl	Day 21
G VI	6	No	-	-	Day 21

3.4 The Experimental Protocol

According to the experiment plan, subgroups of GI and GII with the same observation period (e.g. 3 days GI n=6 and 3 days GII n=6) were treated on the same day.

3.4.1 Application of the intraoral appliance

After the rats were weighed by digital weight scale (Precision balance PFB, KERN & SOHN GmbH, Balingen, Germany), they went under general anaesthesia using a combination of Xylazine hydrochloride (10 mg/kg) and Ketamine hydrochloride (100 mg/kg).

The impression of the maxilla was taken using vinyl polysiloxane impression material (Oran wash L, Zhemarck, Rovigo, Italy) with custom-made impression tray (**Figure 3.1**).

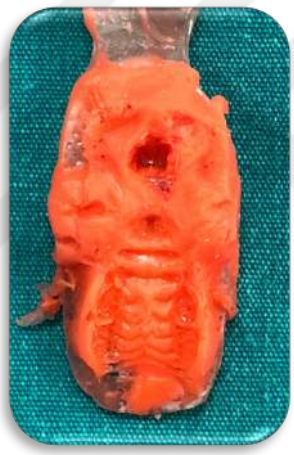

Figure 3.1. Maxillary impression

After rinsing well under running water, the impression was disinfected with Zeta 7 solution (Zhemarck, Rovigo, Italy), and was kept at room temperature for not more than 3 hours for scanning and digital modelling.

After the rat was fixed to table and soft tissues were retracted, the nickel titanium closed coil spring (G&H Orthodontics, Franklin, USA) were used to provide OTM in mesial direction of molar, the spring were fixed to the molar by 0.08 inch ligature wire (G&H Orthodontics, Franklin, USA) and extended to the upper incisors, a hole of 0.5 mm diameter were drilled parallel to long axis of incisors from distal surface at the gingival level to fix the close coil spring by ligature wire to the incisors (231,232). The force of

40 g was measured by dynamometer (Correx, Haag-Streit, Berne, Switzerland) (**Figure 3.2**).

Figure 3.2. Using of intraoral force gauge

In order to stabilize the appliance and prevent soft tissue damage light cured glass ionomer cement (3M UnitekTM Multi-Cure Glass Ionomer Orthodontic Band Cement, Monrovia, California, USA) was placed on the ligature wire end on the posterior and anterior side. No activation of the appliance was made after initial activation (**Figure 3.3**).

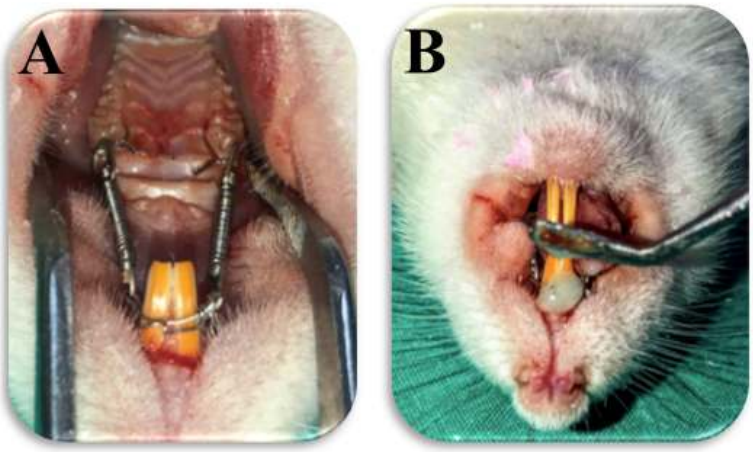

Figure 3.3. Orthodontic appliance in situ: A. Close coil spring B. Glass ionomer cement

The lower incisors were trimmed by metal diamond disk (G&H Orthodontics, Franklin, USA) working by micro drill (Omnidril 35, World Precision Instruments, Sarasota,

Florida, USA) to prevent the breakage of the orthodontic appliance. Because continuous growing of lower incisors in rodents, the trimming procedure was repeated every three days and appliance were checked every day for breakage (**Figure 3.4**).

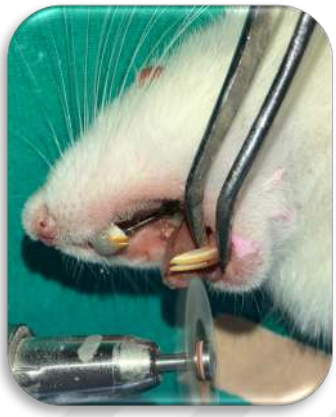

Figure 3.4. Trimming of lower incisors

After placement of the closed coil springs, the injection of HA agent and 0.9% NaCl (Turktipsan, Ankara, Turkey) was made by Hamilton injector (Hamilton Co., Bonaduz AG, Switzerland) to the buccal sulcus of upper 1st molar. The dose was determined as 10µl, which is the maximum amount tissue can accept without overflow (**Figure 3.5**).

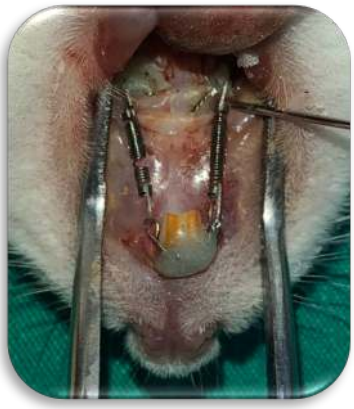

Figure 3.5. Injection of HA into the buccal sulcus

During the experimental period, the general condition of the subjects and their orthodontic appliances were checked every day. The animals were fed pellets broken down like a powder to prevent breakage of the appliance (Korkutelim Animal Food Industry, Antalya, Turkey) and water ad libithum (**Figure 3.6**). Rats with declined general condition or with broken appliance were replaced by new rats of the same

weight and age with repeating all the steps applied before to the excluded rat from the beginning.

Figure 3.6. Soft powdered diet

3.4.2 Sacrification

Immediately after the removal of the appliance from the maxillary jaw of the rats, animal was sacrificed by high dose of anaesthetic solution and the impression was again taken and was maintained for digital scanning on the same day.

3.4.3 Measurement of orthodontic tooth movement

Three digital scanning and OTM measurements were performed using 3Shape Lab Scanner (D-250; 3Shape, Copenhagen, Denmark) and 3Shape Dental System Premium Software Version 19.1 (3Shape, Copenhagen, Denmark) in Smart Gold Dental Laboratory (Gaziantep, Turkey).

In order to determine the clinical values of the study, impressions of the upper jaw were taken at the beginning (T0) and end of the experiment (T1). After scanning, the 3D images were transferred to Dental CAD software (Version 2016.10 Valetta, Exocad, Darmstadt, Germany) for the measurements (**Figure 3.7**).

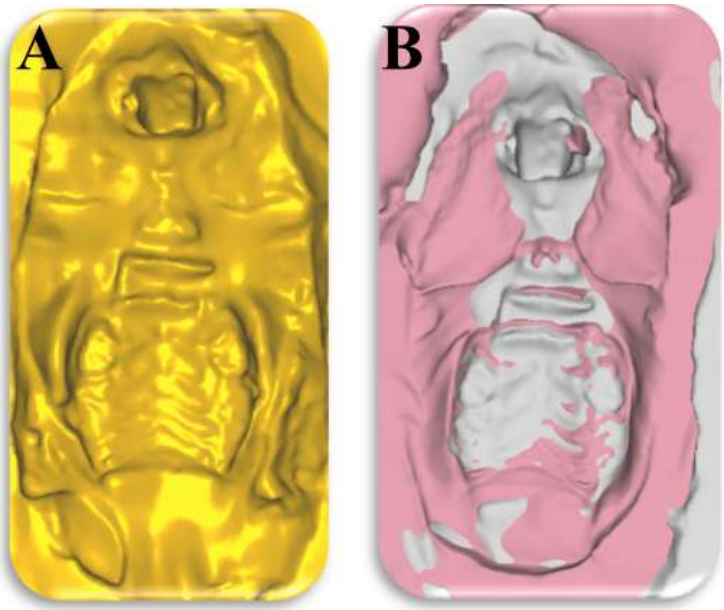

Figure 3.7. 3D model: A. Maxillary impression, B. Superimposition of maxillary impression at T0 and T1

Tooth movement measurements were made on the digital models obtained from the impressions taken at the T0 and T1. The superimposition points were the peaks of the maxillary rugas, which were fixed in front of the 1st molar region. On T0 and T1 models, the first molar mesial marginal ridge midpoint is marked separately and digital models are superimposed. The amount of tooth movement was determined by measuring the distance between them (233) (**Figure 3.8**).

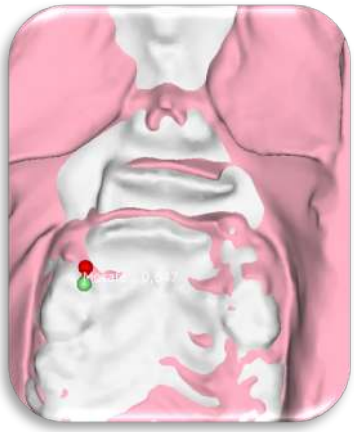

Figure 3.8. Measurement of OTM, green point refer to T0, red point refer to T1

During mesialization of maxillary 1st molar by close coil, axial rotation of the tooth may affect the accuracy of the tooth movement measurements. This angle was formed by two lines, one was the line passed through the distal and mesial contact point of the 1st molar and the other was the line passed on the palatal suture (234). The angle was determined on the digital impression at T0 and T1 and molar rotation amount was calculated (**Figure 3.9**).

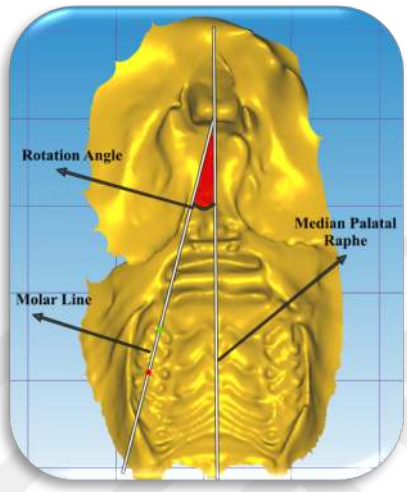

Figure 3.9. Calculation of molar rotation around its long axis

3.5 Histological Evaluation

The immunohistochemical analysis had been performed in Department of Histology and Embryology of Manisa Celal Bayar University.

3.5.1 Tissue Processing

After sacrification of the rats, their maxilla was removed from the skull by surgical scissor and the soft tissues were dissected to expose the underlining bone. Maxilla was seperated into two hemi-maxilla halves along the midpalatal suture by surgical blade (**Figure 3.10**).

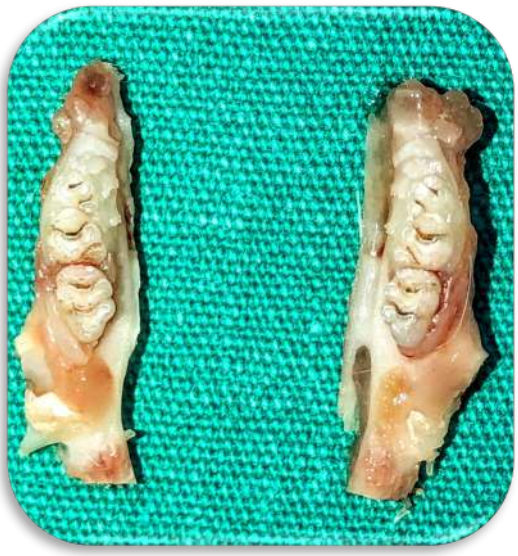

Figure 3.10. Samples of maxilla

Tissue samples were fixed in 10% neutral formaldehyde (Noratex, Istanbul, Turkey) for 2-3 days, after that, samples were washed thoroughly from formalin and decalcification had been done by placing the sample in hydrochloric acid/formic acid solution (Biodec R, Bio-Optica, Milano, Italy), which had been changed once a week for 3 weeks.

Decalcified tissues were dehydrated through alcohol series and then embedded in paraffin blocks that were kept in refrigerator for hardening (**Table 3.2**).

Table 3.2. Steps for preparation of the histological specimen

Procedure	Material used
1. Fixation	% 10 Formalin
2. Decalcification	Hydrochloric acid/formic acid solution
3. Dehydration	Alcohol
4. Clearing	Xylene
5. Infiltration	Paraffin
6. Paraffin embedding	Paraffin

In order to determine on which sections the histological examination will be performed, a pilot study was conducted by cutting a paraffin-embedded tissue block from beginning to end and the area of interest was determined. Sagittal sections from the dental crowns to the root tip of 5 μm thickness were examined microscopically and it was determined that one out of every ten sections would be sufficient for the examination. According to the previous pilot study, the sections were cut 5 μm thick serially and one out of every ten sections was prepared for the slide. Three of the prepared histological slides in each sample, which are the first, middle, and the last ones, were stained with Haematoxylin-eosin (HE) stain, other three slides which are next to HE slides were stained with Masson's Trichrome (MT) stain. Selecting of the slides in immunohistochemical analysis was done as the following matrix: RANKL (First+2, Middle +2, Last –2 slides), OPG (First+3, Middle +3, Last –3 slides), ALP (First+4, Middle +4, Last –4 slides), OCN (First+5, Middle +5, Last –5 slides).

3.5.2 Histological Staining

Selected histological sections were deparaffinized in an oven at 56°C, washed in tap water and stained with HE or MT according to the manufacturer's instruction for histological examination (**Table 3.3**).

Table 3.3. Tissue staining protocol

Procedure	Material used
1. Deparaffinization	Xylene
2. Rehydration	%96 Alcohol, %80 Alcohol, %70 Alcohol, %50 Alcohol
3. Washing	Distilled water
4. Staining	HE,MT
5. Washing	Water stream, %50 Alcohol, %70 Alcohol, %80 Alcohol, %95 Alcohol, Xylene

Stained specimens were observed under a light microscope (Nikon Eclipse E400, Nikon, Tokyo, Japan). For each specimen, the same area was pictured at a

magnification of 100 µm after staining by using a camera. The pictures were then transferred to a computer and calibrated with a Nikon micro meter Slide (Nikon, Tokyo, Japan). All photographs were evaluated with an image analysis program (Clemex Vision Lite Image Analysis3.5; Clemex Technologies, Longueuil, Canada). An area of 1.5 μm^2 in the centre of each section was designated for both the experimental and control group. The number of osteoblasts and osteoclasts were scored in this area of every HE stained slide. Samples for these parameters were scored from +1 (none or minimum) to +5 (maximum) by two histologists who are blind to samples name.

3.5.3 Stereological method

In our study, the volume of the alveolar bone was measured using the Cavalieri principle with the help of Image J (Bone J2 plugin, version 1.5, Wyne Rasband).

The region of interest was determined as the bone between the roots of upper maxillary 1st molar from the lower boundary of the clinical crown to the apical foremen at the root ends. (**Figure 3.11**).

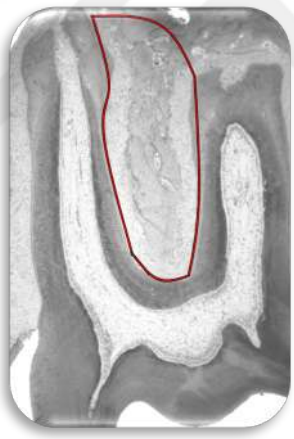

Figure 3.11. The bone area between roots of the maxillary 1st molar

The surface area of each bordered section was calculated and multiplied by thickness of the slide, which was 5 µm to determine the bone volume of each section. Total bone volume of the sample was estimated by multiplying the calculated volumes by the section sampling fraction. The results were evaluated on the bone volume/total volume ratio (BV/TV); which found by the ratio of the volume of alveolar bone (BV) remaining within the borders determined before to the total volume (TV) of all other structures like alveolar bone, periodontal ligament, connective tissue and bone marrow cavities (231).

3.5.4 Indirect immunohistochemical staining

Osteoclast activity was assessed via RANKL antibody detection, while OPG antibody was used for detection of the non-active osteoclasts. ALP antibody was used as an indicator for bone formation and osteoblasts activity. Sections, received OCN antibody, were evaluated to give an idea about the degree of mineralization of the bone.

For immunohistochemical staining, the sections were kept in an oven at 60°C overnight. Clearing the specimen was performed with xylene for 30 min. Then sections were rehydrated with alcohol series decreasing from 95% to 60% and kept in distilled water for 5 min. Sections were kept at room temperature for 15 min in 0.5% trypsin solution confined with Dako (Hydrophobic) pen. To inhibit tissue endogenous peroxidase, 3% hydrogen peroxide was applied for 5 min. Then, sections were washed by phosphate buffer solution 3 times for 5 min every time and immersed in blocking serum for 1h. After blocking serum was removed from the tissue, primary antibodies were incubated overnight. After one day, the section were washed with buffer solution 3 times for 5 min and stained with Streptavidin-Biotin secondary anti-body kit (Zymed Histostain kit San Francisco, USA) for 30 min then washed again with buffered solution for 5 min. In order to fix the immunohistochemical reaction, the sections were stained with (DAB) for 5 minutes. After background staining of the sections with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany), slides had been washed in distilled water for 10 min and preserved in permanent aqueous mounting media (AML060, Scytek, Logan, Utah, USA) (**Table 3.4**).

The stained cells were counted as 0 = negative; (1) = weak positive; (2) = positive; (3 = strong positive. and evaluated with H-score (235),which is a method of assessing the extent of nuclear immunoreactivity. The score is obtained by the formula:

$$\text{HSCORE} = [1 \times (\%\ Cells\ 1+) + 2 \times (\%\ Cells\ 2+) + 3 \times (\%\ Cells\ 3+)]$$

Table 3.4. Steps of immunohistochemical staining

Procedure	Material Used
1. Fixation	%10 Formalin, Paraffin
2. Antigen retrieval	Oven 60°C
3. Clearing	Xylene
4. Rehydration	Alcohol series decreasing from 95% to 60%
5. Blocking	3% H_2O_2, Phosphate buffer
6. Washing	Buffered Solution
7. Primary antibody	RANK, OPG, ALP, and OCN antibodies
8. Washing	Distilled water
9. Secondary antibody (Signal amplification)	Streptavidin-Biotin
10. Chromogenic substrate	DAB
11. Counter stain	Mayer's HE
12. Mounting	Permanent aqueous mounting media AML060

3.6 Statistical Evaluation

The statistical analysis of the histological and clinical results was made by using *Statistical Package for The Social Sciences* version 22.0 software (SPSS Inc., Chicago, IL, USA). In each group, descriptive mean and standard deviation of each parameter were determined. The compatibility of numerical variables to normal distribution was tested with the Shapiro-Wilk test. Paired Student t test was used in comparison of normally distributed variables in two dependent measurements, Unpaired Student t test was used in comparison of normally distributed variables in two independent groups. One-way analysis of variance (ANOVA) test was used for the comparison of parameters between the normally distributed variables in three or more groups; Post-hoc Tukey-kramer and LSD tests were performed for multiple comparisons of normally distributed variables. Kruskal Wallis multiple comparison test was used to compare non-normally distributed variables.

A significance level of $p < 0.05$ was used for all statistical comparisons. The results were evaluated in confidence interval of 95%. The tests were carried out blinding the operator to the groups and time points (by allocating new group codes by another investigator).

4. RESULTS

4.1 Observational Findings

During the experimental period, no infection was observed in rats whose general condition and oral devices were controlled every day. The general health status, food/water consumption, weight loss and stability of the apparatus were checked regularly every day. It was observed that food and water consumption decreased in rats received orthodontic appliance and/or injection compared to the rats in the sham group.

Some rats in the orthodontic appliance groups were found to have problems with the fixation of the apparatus due to the gnawing habits. Five rats because of the broken appliances were excluded from the study and replaced by new rats of the same age and weight with new appliance and the same protocol was applied corresponding to the group deported from.

4.2 Weight Related Results

In term of the weight loss, a significant decrease was observed in groups with orthodontic appliance ($p<0.05$), while a little but not significant weight loss was observed in groups that had received only injection ($p>0.05$). Control group, which was neither treated with injection nor with orthodontic appliance showed a significant weight gain during 21 days of the experiment ($p = 0.002$), (**Table 4.1**).

After placement of the orthodontic appliance, a decrease in the weight of animals was observed apparently in the 3rd day and 7th day, while the loss of weight was less pronounced in the 14th day and 21st day, which may be attributed to adaptation of the animal to the new appliance (**Table 4.2**).

Table 4.1. Intragroup comparison of initial and final weight (mean ± SD) (g)

Group	T0	T1	*p*
GI-1	301.17 ± 21.51	281.34 ± 19.31	0.0004***
GI-3	258.01 ± 63.49	229.17 ± 60.79	0.0003***
GI-7	235.34 ± 35.94	209.84 ± 36.19	0.01**
GI-14	269.84 ± 48.38	251.50 ± 48.92	0.08
GI-21	278.67 ± 35.21	265.50 ± 37.24	0.04*
GII-1	217.67 ± 9.71	199.34 ± 7.71	0.005**
GII-3	222.17 ± 31.32	192.01 ± 30.76	0.0006***
GII-7	231.84 +24.61	203.34 ± 22.26	0.054
GII-14	287.02 +33.50	267.17 ± 33.68	0.002**
GII-21	324.84 +41.62	310.03 ± 43.06	0.021*
GIII	304.84± 34.84	293.17 ± 34.94	0.004**
GIV	340 ± 29.04	335 ± 30.37	0.1
GV	320.17 ±27.21	316.67 ±27.49	0.07
GVI	291.34 ± 19.35	309.5 ± 17.29	0.002**
p	0.127	0.193	-
Total	277.35 ± 50.16	261.68 ± 56.74	0.03*

One way analysis of variance ANOVA test, Significance level p≤0.05, ** p≤0.01, *** p≤0.001*

In term of weight loss, there was no significant difference between GI and GII in the 1st day (p=0.734), 3rd day (p=0.589), 7th day (p=0.343), 14th day (p=0.651), and 21st day (p=0.343). The comparison of weight loss between all study groups revealed a significant difference (p=0.001), because the rate of weight loss and gain were different between rats with and without appliances and/or injection (**Table 4.2**).

Table 4.2. Weight loss in the study animals (mean ± SD) (g)

	Day 1	Day 3	Day 7	Day 14	Day 21
GI	19.83 ± 8.95	28.84 ± 3.97	25.5 ± 5.82	18.33 ± 5.65	13.16 ± 4.02
GII	18.33 ± 5.53	30.16 ± 4.31	28.5 ± 4.55	19.83 ± 5.49	14.83 ± 4.45
GIII	-	-	-	-	11.67 ± 2.34
GIV	-	-	-	-	5.13 ± 2.52
GV	-	-	-	-	3.5 ± 0.54
GVI	-	-	-	-	-18.17 ± 6.89
p	0.734	0.589	0.343	0.651	0.001*

*One way analysis of variance ANOVA test *Significance level p≤0.001*

4.3 Orthodontic Tooth Movement Results

The reliability of OTM measurements was evaluated by intraclass correlation coefficient (ICC), based on the 95% confident interval, values between 0.8 and 0.9 was considered good reliability and higher than 0.9 indicate excellent reliability (**Table 4.3**).

Table 4.3. Evaluation of reliability of method of orthodontic tooth movement

		Intraclass Correlation Coefficient	95% Confidence Interval	
			Lower Bound	Upper Bound
Experiment Side	GI	0.949	0.890	0.976
	GII	0.948	0.892	0.975
	GIII	0.985	0.900	0.998
Control Side	GI	0.968	0.943	0.985
	GII	0.916	0.823	0.960
	GIII	0.988	0.825	0.998

According to the reliability table, the ICC for all groups with tooth movement was found to be above 0.9, which means that the distances measured three times by the same researcher have strong positive correlation between them. This has meant that the method, OTM had measured by, is considered to be reliable and confident (**Table 4.3**).

Because no tooth movement was observed in-group IV, V and VI, these groups were not included in the statistical analysis.

Table 4.4. Orthodontic tooth movement amounts in control and experimental groups (mean ± SD) (mm)

Group	Experiment	Control	*p*
GI-1	0.046 ± 0.009	0.026 ± 0.01	0,002*
GI-3	0.469 ± 0.024	0.341 ± 0.028	p<0.001**
GI-7	0.67 ± 0.024	0.456 ± 0.028	p<0.001**
GI-14	0.989 ± 0.045	0.54 ± 0.047	p<0.001**
GI-21	1.143 ± 0.054	0.93 ± 0.048	p<0.001**
GII-1	0.033 ± 0.008	0.026 ± 0.007	0,06
GII-3	0.45 ± 0.039	0.346 ± 0.026	p<0.001**
GII-7	0.637±0.039	0.444±0.026	p<0.001**
GII-14	0.964±0.067	0.552±0.056	p<0.001**
GII-21	0.977±0.035	0.866±0.037	p<0.001**
GIII	0.99±0.063	0.987±0.061	0,485

*Paired Student t test, Significance level, * p≤0.01, ** p≤0.001*

In GI, there was statistically significant difference between experimental side and control side in all time point (p<0.05), (**Table 4.4**) (**Figure 4.1**).

In GII, measurements of OTM amount illustrated significant difference in all time points (p<0.05) except of the 1st day in which no significant difference was observed (p=0.06), (**Table 4.4**) (**Figure 4.1**).

In GIII, no significant difference was found between experimental and control side after 21 days of experiment (p= 0.485), (**Table 4.4**) (**Figure 4.1**).

Table 4.5. Intragroup comparison of OTM amount (mean ± SD) (mm)

	Group	Day 1	Day 3	Day 7	Day 14	Day 21
Experiment Side	GI	0.046±0.009	0.47±0.024	0.67±0.024	0.989±0.045	1.143±0.054
	GII	0.033±0.008	0.45±0.039	0.637±0.039	0.964±0.067	0.977±0.035
	p	0.009*	0.165	0.07	0.233	<0.001**
Control Side	GI	0.026±0.01	0.341±0.028	0.456±0.028	0.54±0.047	0.93±0.048
	GII	0.026±0.007	0.346±0.026	0.444±0.026	0.552±0.056	0.866±0.037
	p	0.493	0.374	0,133	0.347	0.07

Unpaired Student t test, Significance level p≤0.01, ** p≤0.001*

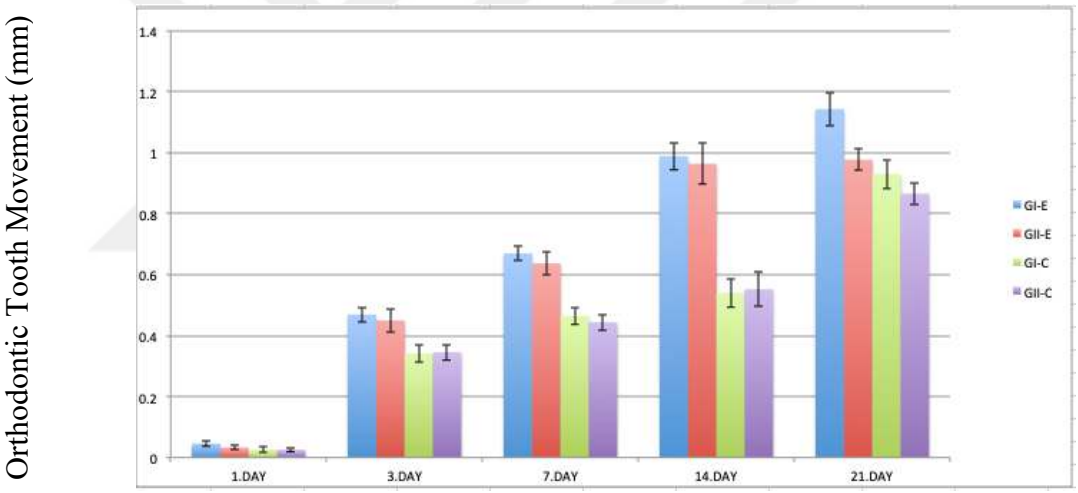

Figure 4.1. Comparison of OTM amount in all time points between GI and GII (E)Experimental, (C)Control

Even though the amount of OTM in the experimental side of GI was a little greater than GII, no significant difference was found between groups (p>0.05) until the 21st day in which GI revealed more significant tooth movement than GII (p<0.001), (**Table 4.5**) (**Figure 4.1**). No significant difference was found between control sides of GI and GII through all time points of the study (p>0.05), (**Table 4.5**) (**Figure 4.1**).

Table 4.6. Multiple comparison of OTM amount between different days

Kruskal Wallis	Experimental Side		Control Side	
Multiple comparison test	GI	GII	GI	GII
3rd.day/7.day	0.0001***	0.001***	0.001***	0.853
3rd.day/14.day	0.0001***	0.0001***	0.005**	0.0001***
3rd.day/21.day	0.0001***	0.0001***	0.0001***	0.0001***
7.day/14.day	0.002**	0.03*	0.001***	0.001***
7.day/21.day	0.0001***	0.001***	0.0001***	0.0001***
14.day/21.day	0.001***	0.022*	0.001***	0.0001***

Significance level p≤0.05, ** p≤0.01, *** p≤0.001*

The mean OTM amount on the 7th day was found to be significantly higher than that was on the 3rd day in GI and GII experimental sides and GII control side (p≤ 0.001), (**Table 4.6**).

The mean amount of OTM on the 14th day was significantly higher than the amount of OTM on the 3rd day and 7th day in both sides of GI and GII (p≤ 0.05), (**Table 4.6**).

The mean amount of OTM on the 21st day was also found to be significantly higher than it were on the 3rd day; 7th day and 14th day in both sides GI and GII (p≤ 0.05), (**Table 4.6**).

In terms of molar angulation, no statistically significant difference was observed between control and experimental sides at T0 (p>0.05). There was no significant difference between control and experimental sides at T1 (p>0.05), (**Table 4.7**).

Table 4.7. Molar angulation degree before and after experiment (mean ± SD) (°)

		Experiment	Control	*P*
Group 1	T0	15.46 ±1.37	16.05±1.4	0.125
	T1	16,82±1.9	16.91±1.89	0.760
	p	0,0001***	0.001***	-
Group 2	T0	15.71±2.56	16.06±1,83	0.480
	T1	16.5±2.38	16.65±2.08	0.606
	p	0.035*	0,007**	-
Group 3	T0	15.4±2.22	16.68±1.36	0.371
	T1	17.13±2.67	17.9±2.31	0.627
	p	0.097	0.089	-

One way analysis of variance ANOVA test, Significance level p≤0.05, ** p≤0.01, *** p≤0.001*

In the comparison between initial and final measurements of molar angle, a high significant difference was found in GI for both control and experimental sides (p≤ 0.001), GII presented a significant increase in molar angles at T1 in respect to T0 in control and experimental sides (p≤ 0.05). However, the amount of molar rotation in GIII was not significant in both the control and experimental side (p>0.05), (**Table 4.7**).

Table 4.8. Intergroup comparison of molar angulation degree at the beginning and end of the experiment (mean ± SD) (°)

		GI	GII	GIII	*P*
Experiment	T0	15.46 ±1.37	15.71±2.56	15.4±2.22	0.895
	T1	16.82±1.9	16.5±2.38	17.13±2.67	0.561
	Angle change %	7.65±8.82	5.72±3.61	11.55 ±8.89	0.357
Control	T0	16.05±1.4	16.06±1,83	16.68±1.36	0.993
	T1	16.91±1.89	16.65±2.08	17.9±2.31	0.641
	Angle change %	5.41±3.76	3.82±2.21	7.12±4.54	0.427

One way analysis of variance ANOVA test

There was no statistical significant difference between experimental side of GI, GII and GIII in term of percentage change in the molar rotation at T0 and T1 ($p>0.05$), (**Table 4.8**).

No statistical significant difference was observed between control sides of GI, GII and GIII in term of percentage change in the molar rotation at T0 and T1 ($p>0.05$), (**Table 4.8**).

4.4 Histological Results

4.4.1 Bone volume results:

As a result of histomorphometric analysis of alveolar bone, no statistically significant difference was found between experimental and control side in GI and GII on days 1, 3, and 7 of the study ($p>0.05$), (**Table 4.9**). However, a statistical significant decrease in bone formation was found in GI and GII at 14.day and 21.day ($p<0.05$). The BV/TV ratio showed no statistically significant difference between right side and left side in GIII, GIV, GV, and GVI of the study ($p>0.05$), (**Table 4.9**).

There was no statistical significant difference in BV/TV ratio between experimental sides of GI and GII at all time points of the study ($p>0.05$) (**Table 4.10**).

No significant difference was observed between control sides of GI and GII at all time points ($p>0.05$), (**Table 4.10**).

There was a statistically significant decrease in BV/TV ratio on day 7 ($p<0.05$), which then increased again significantly on day 21 of the study ($p<0.001$), (**Table 4.10**), (**Table 4.11**).

Table 4.9. BV/TV percentage in experimental and control groups (mean ± SD) (%)

Group	Experimental	Control	*P*
GI-1	47.254 ± 2.528	47.270 ± 2.205	p>0.05
GI-3	43.458 ± 2.44	42.050 ± 1.522	p>0.05
GI-7	29.164 ± 3.846	31.296 ± 3.828	p>0.05
GI-14	25.594 ± 3.562	31.088 ± 3.222	p<0.05*
GI-21	32.111 ± 0.7840	39.890 ± 2.068	p<0.05*
GII-1	47.884 ± 2.340	45.846 ± 2.727	p>0.05
GII-3	46.089 ± 3.447	46.168 ± 3.042	p>0.05
GII-7	26.818 ± 3.910	27.026 ± 3.489	p>0.05
GII-14	26.379 ± 3.757	32.386 ± 3.825	p<0.05*
GII-21	36.071 ± 2.632	39.459 ± 2.028	p<0.05*
GIII	43.271 ± 3.661	42.593 ± 4.392	p>0.05
GIV	43.919 ± 3.744	44.009 ± 3.986	p>0.05
GV	42.340 ± 3.863	41.156 ± 3.379	p>0.05
GVI	41.536 ± 4.163	42.985 ± 2.318	p>0.05

*Paired Student t test, Significance level * p≤0.05*

The BV/TV on day 7 was found to be statistically significant higher than the BV/TV on day 3 in GI and GII both side (p≤ 0.05), (**Table 4.11**).

The BV/TV on day 14 was found to be statistically significant higher than the BV/TV on day 3 in GI and GII both side (p≤ 0.001). However, no significant change in BV/TV was found between day 7 and day 14 (p>0.05), (**Table 4.11**).

The BV/TV in the 21st day was observed to be statistically significant higher than The BV/TV on day 7 and day 14 in GI and GII both side (p≤0.001, p≤ 0.05), respectively (**Table 4.11**).

Table 4.10. Intergroup comparison of BV/TV percentage (mean ± SD) (%)

		Day 1	Day 3	Day 7	Day 14	Day 21
Experiment side	GI	47.25 ± 2.53	43.46 ± 2.44	29.16 ± 3.85	25.59 ± 3.56	32.11 ± 0.78
	GII	47.88 ± 2.34	46.09 ± 3.45	26.82 ± 3.91	26.38 ± 3.76	36.07 ± 2.63
	p	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
Control Side	GI	47.27 ± 2.21	42.05 ± 1.52	31.29 ± 3.83	31.09 ± 3.22	39.89 ± 2.07
	GII	45.85 ± 2.73	46.17 ± 3.04	27.03 ± 3.49	32.39 ± 3.83	39.46 ± 2.03
	p	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05

Unpaired Student t test

Table 4.11. Multiple comparison of BV/TV ratio between different days

Bone/Total Volume %	Experiment Side		Control Side	
Tukey-Kramer Multiple Comparisons Test	GI	GII	GI	GII
3.day/7.day	p<0.05*	p<0.05*	p<0.05*	p<0.05*
3.day/14.day	p<0.001**	p<0.001**	p<0.001**	p<0.001**
3.day/21.day	p<0.0001***	p<0.0001***	p<0.0001***	p<0.0001***
7.day/14.day	p>0.05	p>0.05	p>0.05	p>0.05
7.day/21.day	p<0.001**	p<0.001**	p<0.001**	p<0.001**
14.day/21.day	p<0.001**	p<0.05*	p<0.05*	p<0.05*

Significance level p≤0.05, ** p≤0.001, *** p≤0.0001*

Table 4.12. Relation of OTM with BV/TV percentage

Pearson correlation test				Orthodontic tooth movement
Experimental	GI	Bone/Total Volume %	r	-0,762
			p	0,0001**
	GII	Bone/Total Volume %	r	-0,821
			p	0,0001**
Control	GI	Bone/Total Volume %	r	-0,691
			p	0,004*
	GII	Bone/Total Volume %	r	-0,714
			p	0,002*

*Pearson correlation Test, Significance level * p≤0.01, ** p≤0.001*

In GI experimental side, a significant correlation was observed between BV/TV percentage and tooth movement amounts (r = -0.762, p = 0.0001), (**Table 4.12**).

In GII experimental side, a significant correlation was observed between BV/TV percentage and tooth movement amounts (r = -0.821, p = 0.0001), (**Table 4.12**).

In GI control side, a significant correlation was observed between BV/TV percentage and tooth movement amounts (r = -0.691, p = 0.0001), (**Table 4.12**).
In GII control side, a significant correlation was observed between BV/TV percentage and tooth movement amounts (r = -0.714, p = 0.0001), (**Table 4.12**).

4.4.2 Osteoclast number

In GI, osteoclasts number was increased significantly in the experimental side more than the control side in day 14 and 21 ($p<0.001$), while no significant difference was found in day 1, 3, and 7 ($p>0.05$), (**Table 4.13**).

In GII, osteoclasts number was increased significantly in the experimental side more than the control side in day 14 and 21 ($p<0.05$), while no significant difference was found in day 1, 3, and 7 ($p>0.05$), (**Table 4.13**).

No statistical significant difference in osteoclasts number was found between two side of the animal in GIII, GIV, GV, and GVI ($p>0.05$), (**Table 4.13**).

Table 4.13. Osteoclast number in study groups (mean ± SD) (+1/+5 Score)

	Experimental	Control	*p*
GI-1	1.35 ± 0.17	1.34 ± 0.19	p>0.05
GI-3	2.01 ± 0.32	1.89 ± 0.27	p>0.05
GI-7	3.83 ± 0.41	2.5 ± 0.55	p>0.05
GI-14	5 ± 0.89	2.5 ± 0.57	p<0.001**
GI-21	6.33 ± 0.81	3 ± 0.89	p<0.001**
GII-1	1.41 ± 0.23	1.36 ± 0.21	p>0.05
GII-3	2.13 ± 0.29	1.74 ± 0.27	p>0.05
GII-7	3.17 ± 0.75	2.33 ± 0.52	p>0.05
GII-14	4.67 ± 1.21	2.83 ± 0.75	p<0.05*
GII-21	5.17 ± 1.17	3.33 ± 0.81	p<0.05*
GIII	1.32 ± 0.29	1.21 ± 0.13	p>0.05
GIV	0.91 ± 0.09	0.81 ± 0.11	p>0.05
GV	0.87 ± 0.07	0.62 ± 0.15	p>0.05
GVI	0.89 ± 0.16	0.74 ± 0.19	p>0.05

Paired Student t test, Significance level p<0.05, ** Significance level p≤0.001*

Table 4.14. Intergroup comparison of osteoclast number (mean ± SD) (+1/+5 Score)

		Day 1	Day 3	Day 7	Day 14	Day 21
Experiment side	GI	1.35 ± 0.17	2.01 ± 0.32	3.83 ± 0.41	5 ± 0.89	6.33 ± 0.81
	GII	1.41 ± 0.23	2.13 ± 0.29	3.17 ± 0.75	4.67 ± 1.21	5.17 ± 1.17
	p	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
Control Side	GI	1.34 ± 0.19	1.89 ± 0.27	2.5 ± 0.55	2.5 ± 0.57	3 ± 0.89
	GII	1.36 ± 0.21	1.74 ± 0.27	2.33 ± 0.52	2.83 ± 0.75	3.33 ± 0.81
	p	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05

Unpaired Student t test

There was no significant difference in osteoclasts number between GI and GII experimental side (p>0.05), (**Table 4.14**).

There was no significant difference in new osteoblast formation between GI and GII control side (p>0.05), (**Table 4.14**).

Table 4.15. Multiple comparison of osteoclast number in different days

	Experiment Side		Control Side	
Tukey-Kramer Multiple Comparisons Test	GI	GII	GI	GII
3.day/7.day	p<0.05*	p<0.05*	p<0.05*	p<0.05*
3.day/14.day	p<0.001***	p<0.001***	p<0.001***	p<0.001***
3.day/21.day	p<0.001***	p<0.001***	p<0.001***	p<0.001***
7.day/14.day	p>0.05	p>0.05	p>0.05	p>0.05
7.day/21.day	p<0.01**	p<0.001***	p>0.05	p>0.05
14.day/21.day	p>0.05	p>0.05	p>0.05	p>0.05

Significance level * p≤0.05, ** p≤0.01, *** p≤0.001

The osteoclasts number was increased significantly on day 7 in comparison with day 3 in GI and GII both side ($p \leq 0.05$), (**Table 4.15**).

The osteoclasts number on day 14 was found to be statistically significant higher than that on day 3 in GI and GII both side ($p \leq 0.001$). However, no significant change was found between day 7 and day 14 ($p > 0.05$), (**Table 4.15**).

The osteoclasts number on day 21 was observed to be statistically significant higher than that on day 3 in GI and GII both side ($p \leq 0.001$). However, no significant difference was found in GII on day 21 in comparison with day 7 and 14 (**Table 4.15**) (**Figure 4.2**).

The osteoclast number in GI on day 21 was observed to be higher than that on day 7 ($p < 0.01$), (**Table 4.15**) (**Figure 4.2**).

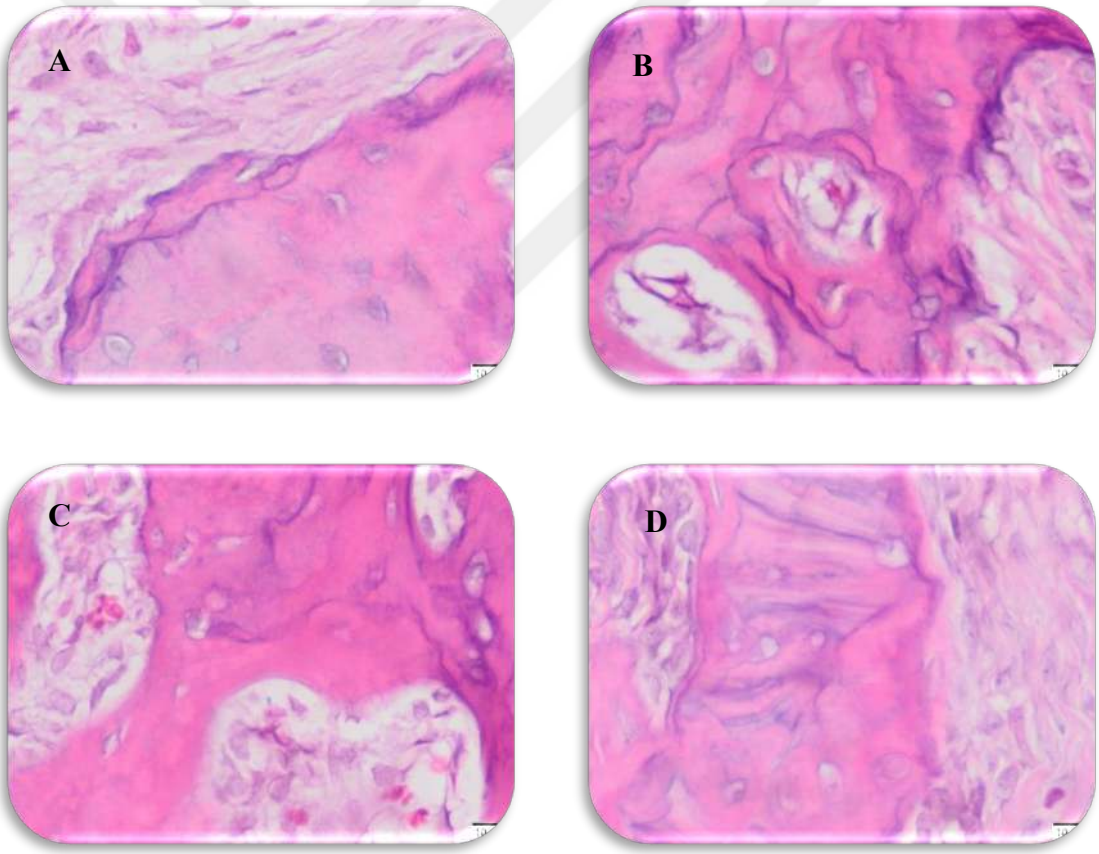

Figure 4.2. Histological sections of alveolar bone on 21.day samples, A: GI experimental side, B: GI control side, C: GII experimental, D: GII control side, Haematoxylin-eosin staining, Scale bar: 400µ, magnification: 10X

4.4.3 New bone cells and capillaries number:

In GI, The number of proliferated bone cells was increased significantly in the experimental side more than the control side in day 14 and 21 ($p<0.05$, $p<0.001$) respectively, while no significant difference was found in day 1, 3, and 7 ($p>0.05$), (**Table 4.16**) (**Figure 4.3**).

In GII, The number of proliferated bone cells was increased significantly in the experimental side more than the control side in day 14 and 21 ($p<0.05$, $p<0.001$), while no significant difference was found in day 1, 3, and 7 ($p>0.05$), (**Table 4.16**) (**Figure 4.3**).

No statistical significant difference in osteoclasts number was found between two side of the animal in GIII, GIV, GV, and GVI ($p>0.05$), (**Table 4.16**) (**Figure 4.3**).

There was no significant difference in new proliferated bone cells number between GI and GII experimental side ($p>0.05$), (**Table 4.17**).

There was no significant difference in new proliferated bone cells number between GI and GII control side ($p>0.05$), (**Table 4.17**).

Table 4.16 New capillaries and bone cells number in study groups (mean ± SD) (+1/+5 Score)

	Experimental	Control	p
GI-1	1.3± 0.27	1.3± 0.32	p>0.05
GI-3	1.36± 0.38	1.4± 0.49	p>0.05
GI-7	3.43± 0.56	3.21± 0.57	p>0.05
GI-14	3.91± 0.62	3.36± 0.39	p<0.05*
GI-21	3.38 ± 0.52	2.25 ± 0.46	p<0.001**
GII-1	1.3 ±0.41	1.3 ± 0.39	p>0.05
GII-3	1.4 ±0.31	1.4 ±0.29	p>0.05
GII-7	3.09 ± 0.49	2.79 ± 0.31	p>0.05
GII-14	4,42 ± 0.52	3.28 ± 0.66	p<0.05*
GII-21	3.8 ± 0.46	2.5 ± 0.52	p<0.001**
GIII	2.38 ± 0.52	2.13 ± 0.64	p>0.05
GIV	1.38 ± 0.52	1.38 ± 0.52	p>0.05
GV	1.38 ± 0.52	1.38 ± 0.52	p>0.05
GVI	1.38 ± 0.52	1.38 ± 0.52	p>0.05

*Paired Student t test, Significance level * p<0.05, ** Significance level p≤0.001*

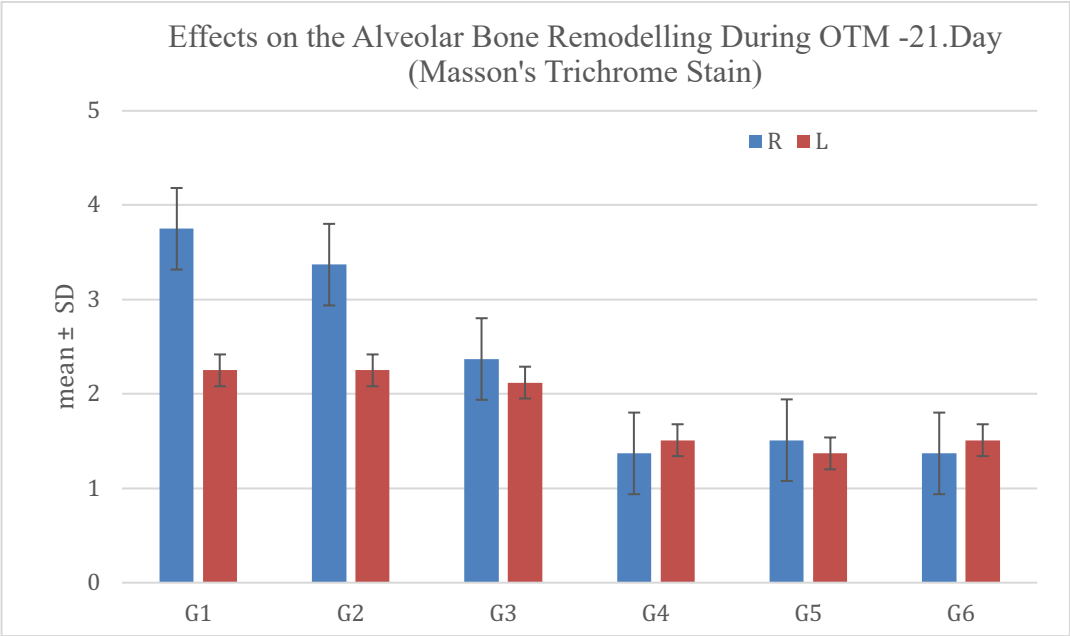

Figure 4.3. Comparison of bone cells number between study groups

Table 4.17 Intergroup comparison of new capillaries and bone cells number in study groups (mean ± SD) (+1/+5 Score)

		Day 1	Day 3	Day 7	Day 14	Day 21
Experiment side	GI	1.3± 0.27	1.36± 0.38	3.43± 0.56	3.91± 0.62	3.38 ±0.52
	GII	1.3 ±0.41	1.4 ±0.31	3.09 ± 0.49	4,42 ± 0.52	3.8 ± 0.46
	p	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
Control Side	GI	1.3± 0.32	1.4± 0.49	3.21± 0.57	3.36± 0.39	2.25 ± 0.46
	GII	1.3 ± 0.39	1.4 ±0.29	2.79 ± 0.31	3.28 ± 0.66	2.5 ± 0.52
	p	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05

Unpaired Student t test

Table 4.18 Multiple comparison of new capillaries and bone cells number in different days

	Experiment Side		Control Side	
Tukey-Kramer Multiple Comparisons Test	GI	GII	GI	GII
3.day/7.day	$p<0.05^{*}$	$p<0.05^{*}$	$p<0.05^{*}$	$p<0.05^{*}$
3.day/14.day	$p<0.001^{***}$	$p<0.001^{***}$	$p<0.001^{***}$	$p<0.001^{***}$
3.day/21.day	$p<0.0001^{***}$	$p<0.0001^{***}$	$p<0.0001^{***}$	$p<0.0001^{***}$
7.day/14.day	p>0.05	p>0.05	p>0.05	p>0.05
7.day/21.day	$p<0.001^{***}$	$p<0.001^{***}$	$p<0.001^{***}$	$p<0.001^{***}$
14.day/21.day	$p<0.001^{***}$	$p<0.01^{**}$	$p<0.01^{**}$	$p<0.01^{**}$

Significance level * *p≤0.05,* ** *p≤0.01,* *** *p≤0.001*

The new bone cells number was increased significantly on day 7 in comparison with day 3 in GI and GII both side ($p \leq 0.05$), (**Table 4.18**).

The new bone cells number on day 14 was found to be statistically significant higher than that on day 3 in GI and GII both side ($p \leq 0.001$). However, no significant change was found between day 7 and day 14 ($p > 0.05$), (**Table 4.18**) (**Figure 4.4**).

The new bone cells number on day 21 was observed to be statistically significant higher than that on day 3,7 and 14 in GI and GII both side ($p \leq 0.001$). (**Table 4.18**) (**Figure 4.4**).

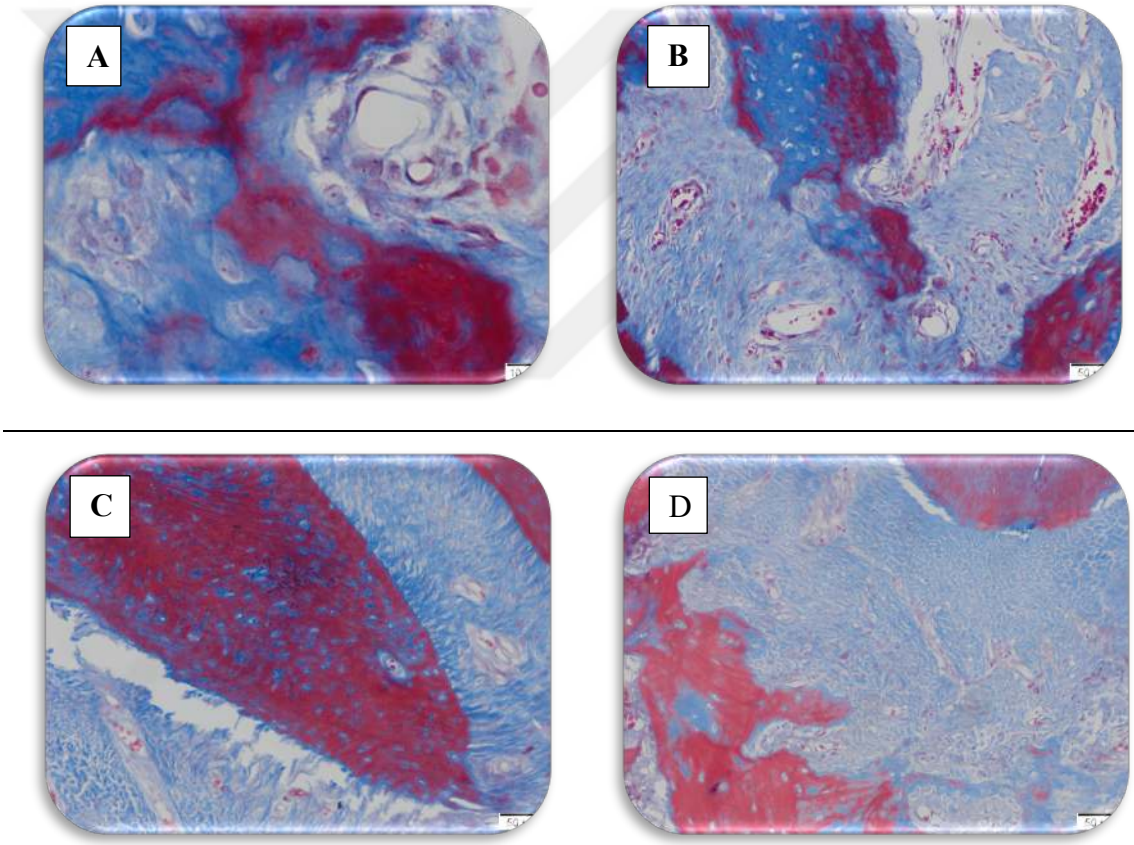

Figure 4.4. Histological sections of alveolar bone on 14.day samples, A: GI experimental side, B: GI control side, C: GII experimental, D: GII control side, Haematoxylin-eosin staining, Scale bar: 400µ, magnification: 10X

4.5 Immunohistochemical Results

4.5.1 RANKL antibodies staining results

RANKL expression was increased significantly in the experimental side of GI and GII in comparison with the control group on day 14 ($p<0.05$), (**Table 4.19**) (**Figure 4.5**).

On the 21st day of the study, the experimental side of the GI and GII showed high significant increase in RANKL expression in comparison to control side ($p<0.001$), (**Table 4.19**) (**Figure 4.5**).

Table 4.19. The expression of RANKL in study groups (mean ± SD) (H Score)

	Experimental	Control	*p*
GI-1	107.24 ± 11.23	104.07 ± 9.26	0.345
GI-3	117.65 ± 13.21	107.86 ± 11.88	0.067
GI-7	126.18 ± 13.21	115.36 ± 12.23	0.084
GI-14	165.41 ± 12.41	128.22 ± 12.43	0.003**
GI-21	223.64 ± 21.11	144.12 ± 18.42	0.001***
GII-1	103.09 ± 13.12	98.93 ± 9.20	0.217
GII-3	121.55 ± 12.49	101.87 ± 11.66	0.047
GII-7	131.28 ± 13.25	109.825 ± 18.06	0.024*
GII-14	177.76 ± 14.63	132.42 ± 12.62	0.002**
GII-21	246.21 ± 15.65	140.87 ± 12.91	p<0.001**
GIII	153.51 ± 12.32	143.42 ± 27.79	0.229
GIV	94.40 ± 18.58	86.14 ± 21.57	0.242
GV	95.77 ± 15.57	84.77 ± 19.13	0.119
GVI	93.019 ± 13.58	96.975 ± 14.88	0.295

Paired Student t test, Significance level p≤0.05, ** p≤0.01, *** p≤0.001*

No significance difference of RANKL expression was found between the two sides of the rat in GIII, GIV, GV and GVI (p>0.05), (**Table 4.19**) (**Figure 4.5**).

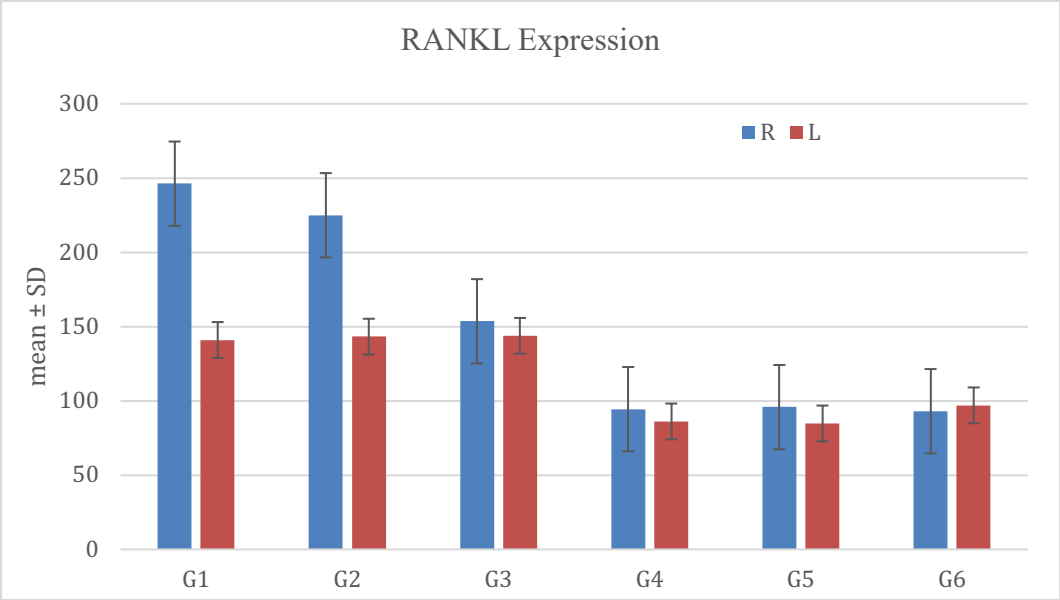

Figure 4.5. Comparison of RANKL expression between study groups

Table 4.20. Intergroup comparison of RANKL expression (mean ± SD) (H Score)

		Day 1	Day 3	Day 7	Day 14	Day 21
Experiment side	GI	107.24±11.23	117.65±13.21	126.18±13.21	165.41±12.41	223.64 ±21.11
	GII	103.09±13.12	121.55±12.49	131.28±13.25	177.76±14.63	246.21±15.65
	p	0.285	0.305	0.306	0.073	0.031*
Control Side	GI	104.07±9.26	107.86±11.88	115.36 ±12.23	128.22±12.43	144.12±18.42
	GII	98.93±9.20	101.87±11.66	109.83±18.06	132.42±12.62	140.87±12.91
	p	0.179	0.199	0.274	0.287	0.372

*Unpaired Student t test, Significance level * p<0.05*

In term of intergroup comparison of RANKL expression, no significance difference was found between experimental sides of GI and GII at all time points (p>0.05), (**Table 4.20**).

No significant difference of RANKL was observed between control groups of GI and GII at all time points (p>0.05), (**Table 4.20**).

Table 4.21. Multiple comparison of RANKL expression

	Experiment Side		Control Side	
Tukey-Kramer Multiple Comparisons Test	GI	GII	GI	GII
3.day/7.day	0.992	0.980	0.993	0.989
3.day/14.day	p<0.001**	p<0.001**	0.232	0.009*
3.day/21.day	p<0.001**	p<0.001**	p<0.001**	p<0.001**
7.day/14.day	p<0.001**	p<0.001**	0.811	0.127
7.day/21.day	p<0.001**	p<0.001**	0.017*	0.007*
14.day/21.day	p<0.001**	p<0.001**	0.567	0.984

*Tukey-Kramer multiple comparison test, Significance level * p≤0.05, ** p≤0.001*

The RANKL expression in GI and GII experimental side was increased significantly on day 21 in comparison to day 3, 7, and 14 (p≤ 0.001), (**Table 4.21**) (**Figure 4.6**).

The RANKL expression in GI and GII control side was increased significantly on day 21 in comparison to day 3 and 7 (p≤ 0.001, p≤ 0.05) respectively, (**Table 4.21**) (**Figure 4.6**).

The RANKL expression on day 14 was found to be statistically significant higher than that on day 3 and 7 in GI and GII experimental side (p≤ 0.001), (**Table 4.15**).

The RANKL expression on day 14 was found to be statistically significant higher than that on day 3 in GII control side (p≤ 0.001), (**Table 4.15**).

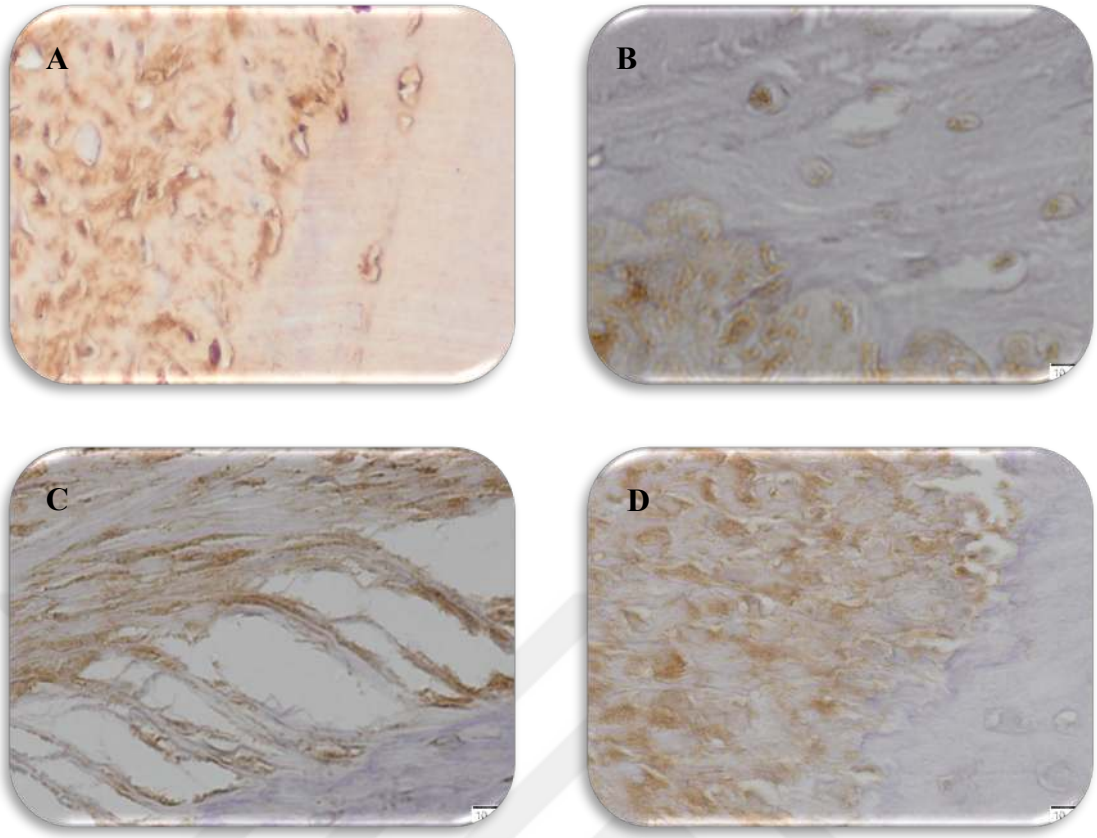

Figure 4.6. Histological sections of alveolar bone on 21.day samples, showing RANKL expression A: GI experimental side, B: GI control side, C: GII experimental, D: GII control side, Scale bar: 400µ, magnification: 10X

4.5.2 OPG antibodies staining results

OPG expression in G1 showed a significant difference between experımental and control side on day 21 ($p<0.001$), (**Table 4.22**) (**Figure 4.7**).

In GII, OPG expression in the experımental side was found to be significantly higher than control side on day 7, 14, and 21 ($p=0.04$, $p=0.03$, $p<0.001$) respectively, (**Table 4.22**) (**Figure 4.7**).

No significant differences of OPG expression were found between two sides in other group ($p>0.05$), (**Table 4.22**) (**Figure 4.7**).

Table 4.22. The Expression of OPG (mean ± SD) (H Score)

	Experimental	Control	*p*
GI-1	99.34 ± 10.8	93.63 ± 12.86	0.231
GI-3	104.04 ± 11.526	99.73 ± 13.17	0.337
GI-7	121.49 ± 14.04	117.4 ± 17.68	0.344
GI-14	125.15 ± 14.01	113.88 ± 16.06	0.068
GI-21	154.37 ± 11.06	119.57 ± 8.43	p<0.001**
GII-1	103.21 ± 15.39	99.42 ± 12.9	0.335
GII-3	102.9 ± 15.72	98.21 ± 11.04	0.257
GII-7	111.03 ± 17.03	120.97 ± 9.96	0.043*
GII-14	139.43 ± 13.86	120.04 ± 8.77	0.031*
GII-21	180.35 ± 19.03	113.84 ± 12.08	p<0.001**
GIII	114.46 ± 17.72	112.97 ± 20.60	0.461
GIV	82.1 ± 25.67	76.3 ± 19.73	0.338
GV	77.33 ± 20.95	71.41 ± 21.37	0.296
GVI	79.56 ± 20.66	77.58 ± 26.98	0.449

*Paired Student t test, Significance level * p<0.05, ** p≤0.001*

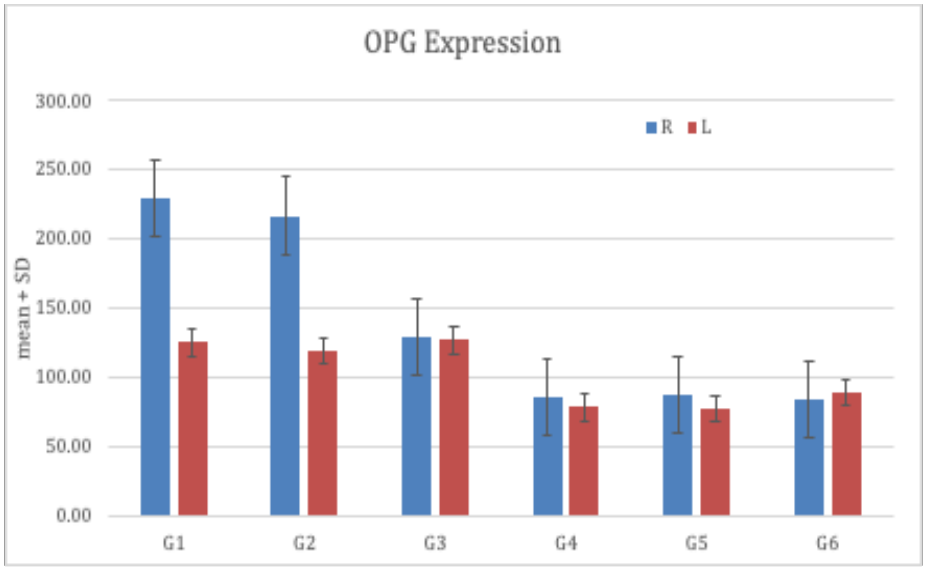

Figure 4.7. Comparison of OPG Expression Between Study Groups

Table 4.23. Intergroup comparison of OPG expression (mean ± SD) (H Score)

		Day 1	Day 3	Day 7	Day 14	Day 21
Experiment side	GI	99.34±10.8	104.04±11.526	121.49±14.04	125.15±14.01	154.37±11.06
	GII	103.21±15.39	102.9±15.72	111.03±17.03	139.43±13.86	180.35±19.03
	p	0.312	0.444	0.136	0.053	0.008*
Control Side	GI	93.63±12.86	99.73±3.17	117.4±17.68	113.88±16.06	119.57±8.43
	GII	99.42±12.9	98.21±11.04	120.97±9.96	120.04±8.77	113.84±12.08
	p	0.227	0.417	0.338	0.215	0.181

*Unpaired Student t test, Significance level * p<0.05*

In term of intergroup comparison of OPG expression, no significance difference was found between experimental sides of GI and GII at all time points (p>0.05), (**Table 4.23**).

No significant difference of OPG was observed between control groups of GI and GII at all time points (p>0.05), (**Table 4.23**).

Table 4.24. Multiple comparison of OPG expression

	Experiment Side		Control Side	
LSD Multiple Comparisons Test	GI	GII	GI	GII
3.day/7.day	0.544	0.993	0.332	0.079
3.day/14.day	0.280	0.002**	0.640	0.107
3.day/21.day	p<0.001***	p<0.001***	0.192	0.505
7.day/14.day	0.998	0.04*	0.912	0.999
7.day/21.day	0.009**	p<0.001***	0.987	0.992
14.day/21.day	0.031*	p<0.001***	0.999	0.997

*Significance level * p≤0.05, ** p≤0.01, *** p≤0.001*

The OPG expression in GI experimental side was increased significantly on day 21 in comparison to day 3, 7, and 14 ($p \leq 0.001$, $p = 0.009$, $p = 0.031$) respectively, (**Table 4.24**) (**Figure 4.8**).

The OPG expression in GII experimental side was increased significantly on day 21 in comparison to day 3 and 7 ($p \leq 0.001$), (**Table 4.24**).

The OPG expression on day 14 was found to be statistically significant higher than that on day 3 and 7 in GII experimental side ($p=0.02$, $p=0.04$), (**Table 4.24**).

The OPG expression on day 14 showed statistically significant difference with that on day 3 and 7 in GI experimental side ($p>0.05$), (**Table 4.24**).

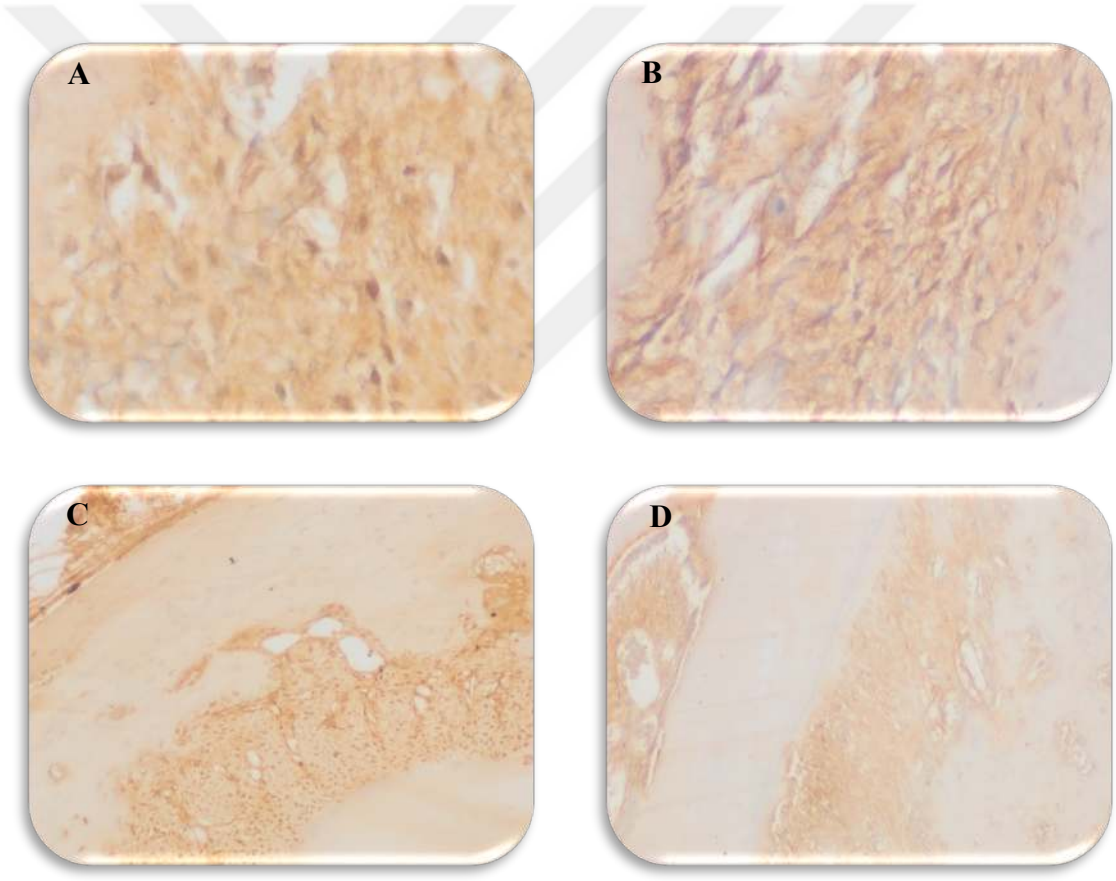

Figure 4.8. Histological sections of alveolar bone on 21.day samples, showing OPG expression A: GI experimental side, B: GI control side, C: GII experimental, D: GII control side, Scale bar: 400µ, magnification: 10X

4.5.3 RANKL/OPG ratio

The RANKL/OPG ratio in experimental side of GI was found to be significantly higher than control side at day 14 and 21 ($p<0.05$), (**Table 4.25**).

The RANKL/OPG ratio in experimental side of GII was found to be significantly higher than control side at day7, 14, and 21 ($p<0.05$), (**Table 4.25**).

No significant difference was found between both sides in other groups ($p>0.05$), (**Table 4.25**).

Table 4.25. RANKL/OPG ratio in study group (mean ± SD)

	Experimental	Control	*p*
GI-1	1.08 ± 0.09	1.14 ± 0.23	0.336
GI-3	1.14 ± 0.16	1.1 ± 0.23	0.387
GI-7	1.06 ± 0.25	1.01 ± 0.23	0.297
GI-14	1.17 ± 0.16	1.14 ± 0.18	0.044*
GI-21	1.45 ± 0.16	1.21 ± 0.15	0.03*
GII-1	1.01 ± 0.15	1.01 ± 0.15	0.481
GII-3	1.21 ± 0.27	1.05 ± 0.16	0.161
GII-7	1.2 ± 0.18	0.91 ± 0.12	0.021*
GII-14	1.28 ± 0.1	1.11 ± 0.14	0.043*
GII-21	1.37 ± 0.12	1.25 ± 0.18	0.048*
GIII	1.36 ± 0.19	1.31 ± 0.36	0.364
GIV	1.26± 0.47	1.26 ± 0.63	0.497
GV	1.28 ± 0.23	1.21 ± 0.31	0.193
GVI	1.21 ± 0.22	1.33 ± 0.31	0.264

*Paired Student t test, Significance level * p<0.05*

Table 4.26. Intergroup comparison of RANKL/OPG ratio (mean ± SD)

		Day 1	Day 3	Day 7	Day 14	Day 21
Experiment side	GI	1.08 ± 0.09	1.14 ± 0.16	1.06 ± 0.25	1.17 ± 0.16	1.45 ± 0.16
	GII	1.01 ± 0.15	1.21 ± 0.27	1.2 ± 0.18	1.28 ± 0.1	1.37 ± 0.12
	p	0.169	0.293	0.138	0.285	0.174
Control Side	GI	1.14 ± 0.23	1.1 ± 0.23	1.01 ± 0.23	1.14 ± 0.18	1.21 ± 0.15
	GII	1.01 ± 0.15	1.05 ± 0.16	0.91 ± 0.12	1.11 ± 0.14	1.25 ± 0.18
	p	0.141	0.317	0.183	0.362	0.345

Unpaired Student t test

In term of intergroup comparison of RANKL/OPG ratio, no significance difference was found between experimental sides of GI and GII at all time points (p>0.05), (**Table 4.27**).

No significant difference of RANKL/OPG ratio was observed between control groups of GI and GII at all time points (p>0.05), (**Table 4.27**).

Table 4.27. Multiple comparison of RANK/OPG ratio

	Experiment Side		Control Side	
LSD Multiple Comparisons Test	GI	GII	GI	GII
3.day/7.day	0.456	0.909	0.119	0.113
3.day/14.day	0.077	0.496	0.286	0.486
3.day/21.day	0.008**	0.119	0.0427*	0.031*
7.day/14.day	0.015*	0.427	0.391	0.027*
7.day/21.day	0.001***	0.096	0.001***	0.001***
14.day/21.day	0.320	0.365	0.219	0.125

*Significance level * p<0.05, ** p≤0.01, *** p≤0.001*

The RANK/OPG ratio in GI experimental side was increased significantly on day 21 in comparison to day 3, 7 (p= 0.001, p= 0.008) respectively (**Table 4.27**) (**Figure 4.9**).

The RANK/OPG ratio on day 21 was found to be statistically significant higher than that on day 3 and 7 in GI and GII experimental side (p<0.05), (**Table 4.27**) (**Figure 4.9**).

The RANK/OPG ratio on day 14 showed statistically significant difference with that on day 3 and 7 in GI experimental side (p=0.015, p=0.077), (**Table 4.27**) (**Figure 4.9**).

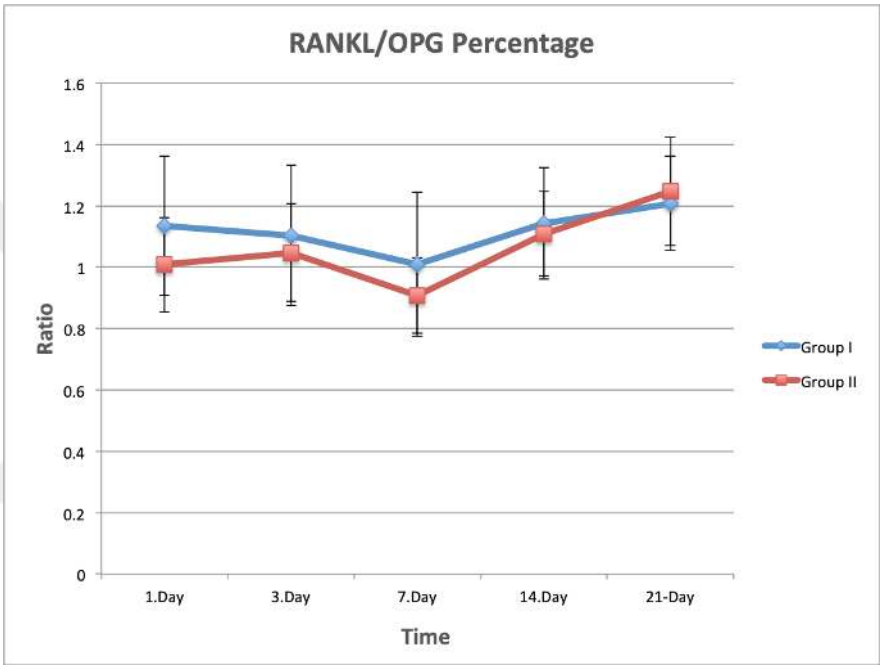

Figure 4.9. RANKL/OPG ratio at experimental side

4.5.4 ALP antibodies staining results

ALP expression was increased in experimental side of GI and GII significantly in comparison with control side on day 14 (p<0.05) and decreased again on day 21 with no significant difference with control side on day 21 (p>0.05), (**Table 4.28**).

No significant difference was observed between the both sides in other groups (p>0.05), (**Table 4.28**).

Table 4.28. The expression of ALP in study groups (mean ± SD) (H Score)

	Experimental	Control	*p*
GI-1	101.27 ± 34.19	93.04 ± 19.53	p>0.05
GI-3	109.91 ± 27.06	107.74 ± 34.52	p>0.05
GI-7	157.17 ± 31.25	127.91 ± 39.15	p>0.05
GI-14	173.58 ± 29.56	117.25 ± 27.01	p<0.05*
GI-21	143.96 ± 49.59	111.39 ± 29.46	p>0.05
GII-1	103.93 ± 22.13	101.27 ± 28.91	p>0.05
GII-3	115.47 ± 31.45	107.85 ± 29.17	p>0.05
GII-7	153.27 ± 33.87	123.43 ± 33.09	p>0.05
GII-14	167.31 ± 39.41	114.51± 19.62	p<0.05*
GII-21	149.74 ± 54.79	117.47 ± 22.15	p>0.05
GIII	129.30 ± 34.56	118.39 ± 47.84	p>0.05
GIV	94.39 ± 35.53	86.14 ± 32.42	p>0.05
GV	95.77 ± 36.05	84.77 ± 31.91	p>0.05
GVI	93.02 ± 35.012	88.89 ± 33.46	p>0.05

*Paired Student t test, Significance level * p<0.05*

Table 4.29. Intergroup comparison of ALP (mean ± SD) (H Score)

		Day 1	Day 3	Day 7	Day 14	Day 21
Experiment side	GI	101.27±34.19	109.91±27.06	157.17±31.25	173.58±29.56	143.96±49.59
	GII	103.93±22.13	115.47±31.45	153.27±33.87	167.31±39.41	149.74±54.79
	p	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
Control Side	GI	93.04±19.53	107.74±34.52	127.91±39.15	117.25±27.01	111.39±29.46
	GII	101.27±28.91	107.85±29.17	123.43±33.09	114.51±19.62	117.47±22.15
	p	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05

Unpaired Student t test

In term of intergroup comparison of ALP expression, no significance difference was found between experimental sides of GI and GII at all time points (p>0.05), (**Table 4.29**).

No significant difference of ALP was observed between control groups of GI and GII at all time points (p>0.05), (**Table 4.29**).

Table 4.30. Multiple comparison of ALP

	Experiment Side		Control Side	
Kruksal-Wallis Multiple Comparisons Test	GI	GII	GI	GII
3.day/7.day	p>0.05	p>0.05	p>0.05	p>0.05
3.day/14.day	p<0.05*	p<0.05*	p>0.05	p>0.05
3.day/21.day	p<0.001**	p<0.001**	p>0.05	p>0.05
7.day/14.day	p>0.05	p>0.05	p>0.05	p>0.05
7.day/21.day	p>0.05	p>0.05	p>0.05	p>0.05
14.day/21.day	p>0.05	p>0.05	p>0.05	p>0.05

Significance level * $p \leq 0.05$, ** $p \leq 0.001$*

The ALP expression in GI and GII experimental side was increased significantly on day 21 in comparison to day 3 ($p \leq 0.001$), (**Table 4.30**) (**Figure 4.10**).

The ALP expression on day 14 was found to be statistically significant higher than that on day 3 in GI and GII experimental side ($p \leq 0.05$), (**Table 4.30**) (**Figure 4.10**).

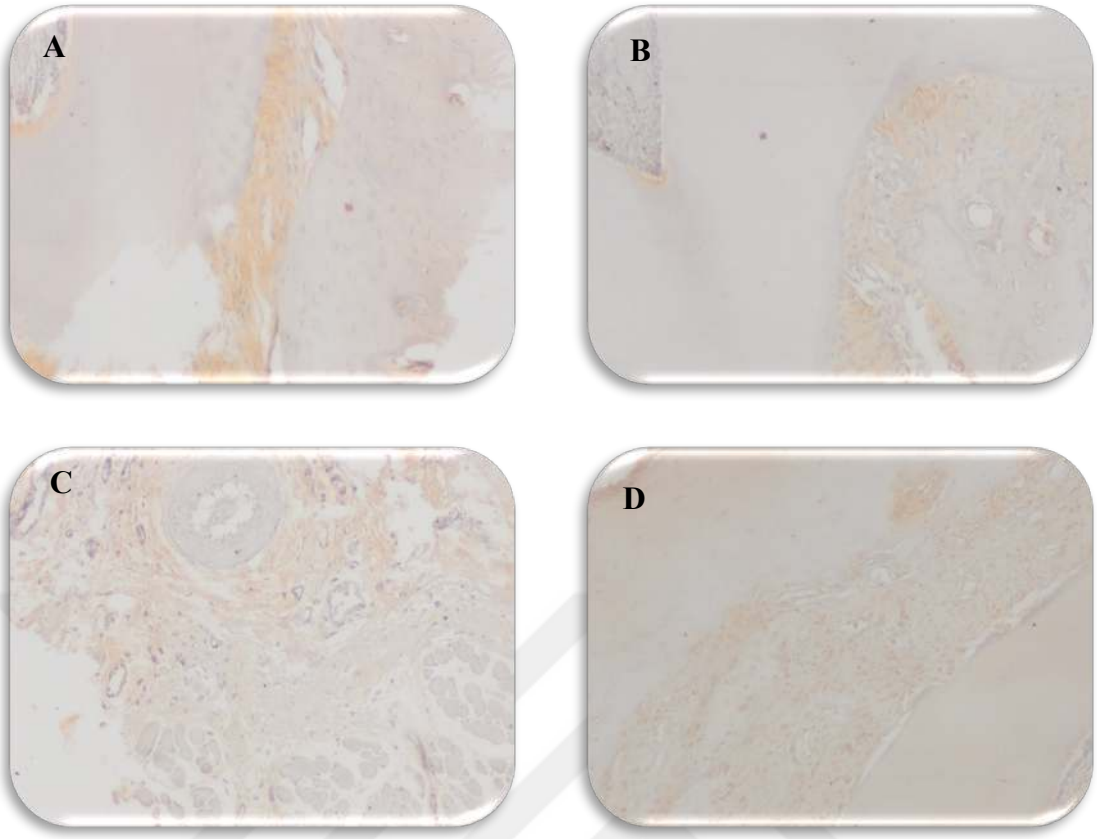

Figure 4.10. Histological sections of alveolar bone on 21.day samples, showing ALP expression A: GI experimental side, B: GI control side, C: GII experimental, D: GII control side, Scale bar: 400µ, magnification: 10X

4.5.5 OCN antibodies staining results

No significant difference in OCN expression was observed between experimental and control side in all study groups ($p>0.05$), (**Table 4.31**).

Table 4.31. The expression of OCN (mean ± SD) (H Score)

	Experimental	Control	*p*
GI-1	117.19 ± 39.65	107.76 ± 31.76	p>0.05
GI-3	122.61 ± 29.17	125.71 ±29.07	p>0.05
GI-7	148.73 ± 39.65	129.29 ±37.85	p>0.05
GI-14	174.38 ± 27.06	143.78 ±34.98	p>0.05
GI-21	191.62 ± 42.71	127.30 ± 48.12	p>0.05
GII-1	109.74 ± 33.21	111.63 ± 34.04	p>0.05
GII-3	121.58 ± 27.61	119.75 ± 29.54	p>0.05
GII-7	166.96 ±22.19	126.68 ± 23.17	p>0.05
GII-14	183.21 ±33.56	154.76 ± 22.81	p>0.05
GII-21	205.16 ± 23.21	148.79 ± 32.42	p>0.05
GIII	145.46 ± 45.71	135.30 ± 51.14	p>0.05
GIV	111.56 ± 35.53	101.81 ± 32.42	p>0.05
GV	113.18 ± 36.05	92.475 ± 32.95	p>0.05
GVI	109.93 ± 35.01	96.975 ± 34.56	p>0.05

Paired Student t test

Table 4.32. Intergroup comparison of OCN (mean ± SD) (H Score)

		Day 1	Day 3	Day 7	Day 14	Day 21
Experiment side	GI	117.19±39.65	122.61±29.17	148.73±39.65	174.38±27.06	191.62±42.71
	GII	109.74±33.21	121.58±27.61	166.96±22.19	183.21±33.56	205.16±23.21
	p	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
Control Side	GI	107.76±31.76	125.71±29.07	129.29±37.85	143.78±34.98	127.30±48.12
	GII	111.63±34.04	119.75±29.54	126.68±23.17	154.76±22.81	148.79±32.42
	p	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05

Unpaired Student t test

In term of intergroup comparison of OCN expression, no significant difference was found between GI and GII experimental side (p>0.05), (**Table 4.32**).

In term of intergroup comparison of OCN expression, no significant difference was found between GI and GII control side (p>0.05), (**Table 4.32**).

Table 4.33. Multiple comparison of OCN values

	Experiment Side		Control Side	
LSD Multiple Comparisons Test	GI	GII	GI	GII
3.day/7.day	p>0.05	p<0.05*	p>0.05	p>0.05
3.day/14.day	p<0.05*	p<0.05*	p>0.05	p>0.05
3.day/21.day	p<0.001**	p<0.001**	p>0.05	p>0.05
7.day/14.day	p>0.05	p>0.05	p>0.05	p>0.05
7.day/21.day	p>0.05	p>0.05	p>0.05	p>0.05
14.day/21.day	p>0.05	p>0.05	p>0.05	p>0.05

Significance level $^{*}p\leq0.05$, $^{**}p\leq0.001$

The OCN expression in GI and GII experimental side was increased significantly on day 21 in comparison to day 3 (p≤ 0.001), (**Table 4.33**) (**Figure 4.11**).

The OCN expression on day 14 was found to be statistically significant higher than that on day 3 in GI and GII experimental side (p≤ 0.05), (**Table 4.33**).

The OCN expression on day 7 was found to be statistically significant higher than that on day 3 in GII experimental side (p≤ 0.05), (**Table 4.33**).

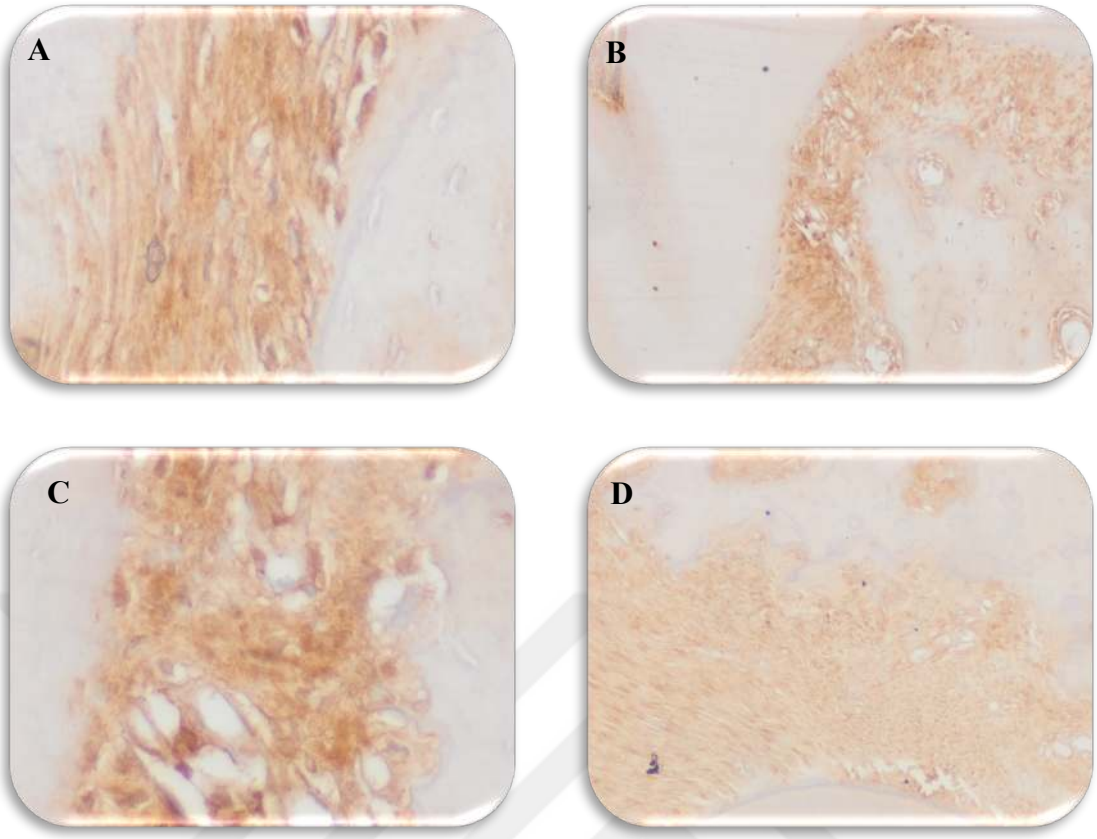

Figure 4.11. Histological sections of alveolar bone on 21.day samples, showing OCN expression A: GI experimental side, B: GI control side, C: GII experimental, D: GII control side, Scale bar: 400µ, magnification: 10X

5. DISCUSSION

In this study, we evaluated the clinical and histomorphometric changes in the alveolar bone after application of orthodontic force with local injection of HMWHA or LMWHA in rats.
The aim of the current study was to accelerate OTM by administration of a compound that is biocompatible, safe, have no systemic effects, and less traumatic than other surgical and non-surgical procedures.

Acceleration of the OTM will shorten the total treatment time that is a very important factor for orthodontist and patients to decrease the adverse effects associated with prolonged orthodontic therapy like root resorption, caries, and loss of paradental tissue (5,236). Reducing the time span of orthodontic treatment attracted many researchers; so many methods of acceleration had been explored. Some accelerating procedures were aggressive for the patient as including surgical operations, while others had not practical, expensive, systemic harmful, or need a patient cooperation (76).

The fundamental of the orthodontic therapy is the bone remodelling process so any substance that could increase the rate of alveolar bone remodelling would supposed to enhance the OTM and vice versa (133). There is a cascade of biological events happened at a cellular and molecular level in response to the orthodontic force. Proliferation, migration and activation of certain bone cells occurred at the site of force application; this is done by secretion of endogenous biological substances such as inflammatory mediators, cytokines and neurotransmitters (9). The information obtained from the biology of tooth movement studies lead the researchers to target the mediators in the pathways of bone remodelling by exogenously applied substances that act as a conductive of endogenous mediators (237).

The ability of osteoclasts to resorb bone is depending on the binding to the bone surface through specific cell adhesion proteins like osteopontin (238), Osteoclasts have cell adhesion proteins that are able of binding to HA such as CD44 binding protein, which could act alone or in combination with other matrix proteins to enhance osteoclast adhesion to the bone surface (239,240).

In the light of previous findings, several studies had been conducted to investigate the role of HA in bone resorption, some of these studies demonstrated a strong link between HA synthesis and bone resorption. In their in-vitro study, Luben et al. found that the endogenous HA synthesis induced by parathyroid extract was found to increase calcium release from bone in a parallel manner (241). Cao et al. observed that addition of exogenous HA to bone marrow stem cells culture improved the expression of RANKL in bone that in turn reflected a higher osteoclastic activity (242). Ariyoshi et al. found in his study that different molecular weight of HA had contrasting effects on osteoclasts, so LMWHA enhanced the osteoclast formation, while HMWHA had no effect on osteoclast differentiation according to his study (243). Fujii et al. concluded in his study that the interactions of HA with CD44 protein found on osteoblasts and osteoclasts might play an essential role in bone metabolism, including osteoclastogenesis (244). As an adjunct to previous information, it was found that genetic deficiencies of β-glucuronidase, an enzyme responsible of HA degradation, in Sly Syndrome may cause resorptive deformity in the skeleton (245).

These data strongly linked induction of HA synthesis to osteoclastic resorption, prompting us to suggest that an increase of HA level in extracellular matrix could enhance bone resorption which is the key event in accelerated orthodontic treatment.

To evaluate the effect of HA on alveolar bone remodelling and OTM, an animal orthodontic model had been preferred as a preclinical trial. The remarkable anatomical and physiological similarities between human and animals, particularly mammals, lead the researchers to assess their investigations in animals before application in human. In literatures concerned with orthodontic, a large number of animal species had been used such as rats, mouse, guinea pigs, rabbits, dogs, cats, and monkeys (246), but the most important factor in animal studies is to consider the variations between different species and to achieve results that can be comparable to human experiments.

As most of the orthodontic studies used rats as a research model, the differences between the rat and human teeth, alveolar bone and PDL have to be considered, rat teeth are very tiny and diminutive, a rat molar is approximately 50 times smaller than a human molar, rats have 16 teeth 3 molars and 1 incisor on each side (247). In terms of the paradental structure, Reitan and Kvam found that the alveolar bone in rats is denser with lack of bone marrow spaces and osteons. The calcium metabolism is controlled by

intestinal absorption rather than the bone tissue itself. To some extent variations in the periodontal fibers also exist between human and rats as no elastic fibers had been seen in periodontal ligament space and moderate number of elastic fibers had been found in supra alveolar tissue, while well developed elastic fibers had been existed in supra alveolar crest of human (248). The bone turnover of rats is also faster than that of humans; hyalinization in human is started after thirty to forty hours while in rats it is started more rapidly after six hours only (249). It was stated that PDL space was diminished after application of long-term heavy forces with delayed bone deposition because of the lack of osteoid tissue (250).

Despite of the previous differences in teeth size and paradental structures, rats are generally considered to be a good model to study OTM, because of several advantages; rats are relatively inexpensive, most of the antibodies required for immunohistochemical analysis are available for mice and rats, and application of an effective orthodontic appliance is easier in rats than smaller rodents like mice (251).

Molar teeth are more preferred than incisors for the orthodontic studies because rat incisors have a completely different morphology with an open apex for total life that helps the tooth to continue eruption. Besides, mesial movement is the most comparable to human tooth movement because on the buccal side small amount of compact bone exist (251). One of the important factors that should be considered during OTM in rats is the continuous eruption of incisors and the physiological molar distal drift that could affect the result of OTM (246). Male rats were more suitable to the orthodontic studies in order to eliminate the hormonal effects of estrous cycle on bone turnover.

A split mouth design was used in this study to decrease the possible bias related to the individual variations between the rats, which may lead to different response to the treatment. Even that the main comparison was between right and left sides of the same rat, multiple intergroup comparisons were included in the study to display the effect of HA on bone turn over in different rats that reflect the diversity between orthodontic patients. Various groups with or with out OTM and/or injection were included to counteract the previously mentioned physiological drift and incisors eruption (246).

Another important determinant in animal studies is the age of the animal, because of the alteration in the bone metabolism at different age. Misawa et al. revealed that under

normal physiological conditions, the rate of alveolar bone remodelling decreased rapidly between 10-weeks old and 30, 50 and 80-weeks old rats (252). Since younger animals showed more physical tolerance to the research conditions, 10-weeks old adult rats were preferred in the present study, both for the ease of working in the mouth and more adaptation to the orthodontic appliance than older rats.

Literature review on experimental tooth movement reveals that experimental tooth movement could be done by utilizing several rat models: Waldo's rat model which used elastic rubber band placed between 2nd molar and 1st molar (253), King's rat model which applied close coil spring to 1st molar with using incisors teeth as an anchorage unit to deliver continuous force to the molar tooth. King's rat model is a reliable, sensitive, and valid technique for quantifying mesial OTM using tipping forces on the rat molar (254). In addition to previous models, another less frequent model uses expansion spring between upper molars to investigate the tooth movement in buccal direction (255,256) In this study, a constant and continuous force was delivered by nickel titanium closed coil springs as a modification of King's orthodontic rat model, which are proved to be effective in delivering such a force by only one activation (251).

The magnitude of the force applied to molar teeth varies between various studies ranging from 10 g to more than 100 g. Lee et al. used 100 g in his study (257); Sodagar et al preferred 60 g of force for orthodontic movement of rat's molar (258). Tsai et al. applied force of 50 g (259), while Gulec et al. used 40 g of force for mesialization molar teeth (153). King et al. reported that the force to be applied for effective molar tooth movement in rats should be between 20-40 g, forces over 40 g can also be applied, but increasing the magnitude of the applied force would not affect the rate of the tooth movement (254). Some researchers tend to choose lighter force such as 10 g or 25 g (19,231). In his study, Kohno et al. used the forces even lighter than 10 g and found that the tooth movement induced by light forces are close to physiological movements (260). Nakano et al. revealed in his study that 10, 25 and 50 g force applications provided greater orthodontic tooth movement than 100 g (261). As the majority of the researchers considered using lighter forces between 10 g and 50g, a continuous force of 40 g was delivered to maxillary 1st molars in our study, because it is considered easier to measure by force gauge during the procedure (26,132,153,233,262,263).

The duration of the experiment is related to great extent into the cycle of the bone metabolism of rats. Ren et al. found in a systematic review done on 2004 that 40% of the studies had been done for less than 7 days, 31% for 7 to 14 days, and 18% evaluate the tooth movement over a longer duration from 14 to 28 days. Taking the previous systematic review into consideration, the experiment duration of longer than 14 days is considered more reliable in terms of assessing bone turnover rate (251).

When the current literature was examined, the duration of experiments ranges from 3 days to 12 weeks in tooth movement studies on rats. Most of the elastics used studies were not continued for more than 7 days because the loss of the elasticity of rubber bands used in these studies (23). Besides, most of close coil spring used studies continued for several time points even more than 28 days, because the continuous constant force delivered by the spring (19,148,153). In our study, five different experimental time were planned: 1 and 3.days group as early follow-up group, 7, 14 and 21 days for follow-up of bone turn over in the late phase of OTM as in Alazzawi et al. study and Balloul et al. study (19,264).

After placement of the orthodontic appliance, the psychological stress encountered during the procedure lead to significant weight loss at first, which decreased with time as the rat returned to the normal eating habits after adaptation to the appliance. The resulting psychological stress may affect the OTM (265), so the food were delivered to the animals after breaking down to powder to facilitate the normal eating.

Various molecular weight of HA compounds have been used in a variety of medical applications to produce different biological effects. Two basic types were used in most of the HA preparations the HMWHA that have a molecular weight more than 1000 kDa and the LMWHA that have a molecular weight less than 1000 kDa (266). Yang et al. observed in his study that LMWHA promote cell adhesion better than HMWHA (267).

The different cellular response to different size of HA had been evaluated by several in-vitro, in-vivo and clinical studies, it was found that the optimum weight for osteoarthritis treatment range from 500 - 4000 kDa (268), the preferred molecular weight fro ophthalmic applications was 600 - 1000 kDa because of their physico-chemical and biological properties, tolerance and handling (269). Haung et al. revealed that the HA had a molecular weight-specific mode of action on bone formation with 900

– 2300 kDa enhanced bone formation better than 60 kDa HA (270). The effect of HA with two molecular weight 1800 kDa and 1000 kDa revealed an improving of probing depth and clinical attachment level after scaling and root planning in periodontitis patient (271). Ballini et al. suggested that autologous bone combined with an esterified low-molecular HA preparation seems to have good capabilities in accelerating new bone formation in infrabony defects (272). In term of maxillary expansion, the HMWHA (5000 kDa) increased the rate of bone formation in expanded intermaxillary suture, while LMWHA (645 kDa) had no effect (42). In our study we investigated the effect of HMWHA with 6000 kDa molecular weight and LMWHA with 750 kDa molecular weight on the bone resorption after OTM.

In the OTM studies, the measurement of OTM had been done in different methods: using of high accurate calliper intraorally (23,165,252,262), on plaster model (148,273,274) or on dissected maxilla (275). Digital or manual tracing of impressions or photographs of plaster models is another method for measurement of OTM (19,165,276) Some researchers utilize X-ray for more accurate measurement of OTM; such as two-dimensional Cephalostat (277), two-dimensional radiographic Faxitron sample imaging (264), or three-dimensional micro computed tomography (234,278). Usage of the histological section for measuring the amount of tooth displacement via other structures in the slide was reported in one of the studies (263). Recent literatures reported using of 3D Scanning of impressions or plaster models and superimposition of initial and final records (153,233)

Every method previously mentioned had some limitations as most intraoral or on models measurement methods could not use a stable point. Some of them calculated the distance between 1st molar mesial cusp tip and incisors, while other used 1st molar distal cusp tip and 2nd molar mesial cusp tip which are not accurate methods because of the continuous eruption of incisors and the physiological 2nd molar distal drift or mesial migration by transseptal fibers affected by 1st molar mesialization were not considered in these methods (133). Estimating of the OTM amount by micro tomography can be considered very accurate but the expensiveness of such a method restricted its use. To measure the OTM accurately in our study, maxillary impressions were taken from the rats by polysiloxane impression material at the beginning and at the end of the experiment. Afterwards, the impressions were transferred into digital three-dimensional model by 3D lab scanner and the models of the initial and final impression of each rat

had been superimposed, then the measurements points were determined as the midpoint of the mesial marginal ridge of 1st molar that is easy to locate.

To obtain precise values of OTM, measurements were repeated three times by the same researcher who is blind to the groups name by coding the names by other researcher; the measurements made at different times were found to have strong correlation with each other.

For better understanding of the mechanism of action of HA on bone remodelling during OTM, histological inspection of the tissue sections obtained from the rat maxilla mesial to the 1st molar had been examined. The estimation of bone volume density after OTM had been utilized in several tooth movement studies to evaluate the amount of resorbed bone. Two main techniques were used for calculating the bone parameters: the stereological method and the micro tomography method (231,233,279). Gulec et al calculated the BV/TV ratio in the interradicular area of 1st molar by stereological analysis to measure alveolar bone density (153). Meh et al. estimated the bone volume density around the five roots of 1st molar by point counting stereological method (231). Other studies in the literature used micro computed tomography to measure the bone parameter like volume and density (233,279). In our study, the stereological analysis of the bone tissue was used for interpretation of 3 dimensional bone parameters from 2 dimensional sections due to some limitation of micro tomography like artefacts associated with radiation (280), the difficulty in achieving standard position for scanning of animals (281), and the effect of radiation dose on bone metabolism that found to be loss about 30% of BV/TV ratio in mice (282).

When we examined the weight related results of the experimental animals, a continuous weight loss was illuminated from the 1.day and increased through day 3 and 7 to decrease again in the following days and these finding were corresponding with outcomes of other studies of experimental OTM in rats (283,284). Some experiments with different study design or appliance type showed weight gain after 21 days of experiment (165) or in the second week (277). The weight loss seen in our study may be due to the difficulty of feeding with orthodontic appliance in situ especially that split mouth design with orthodontic appliance in both sides were used, it is seen that the amounts of weight loss gradually decrease towards the end of the experiment, which shows us that our subjects can tolerate to some extent the double-sided study model.

The amount of OTM showed a high significant difference between experiment side and control side in the groups received adjunctive HA injection with OTM through out all the study days, these findings revealed a high ability of HA to fasten OTM. Even that the HA of both high and low molecular weight increased the OTM significantly, HMWHA accelerated the OTM in a rate higher than LMWHA on day 21, this may be attributed to the more enzymatic events required for HMWHA to be cleaved to smaller size oligomer, Thus extend the effect of HA on bone turnover (285). Previous study by Sadikoglu et al. found that HMWHA increase bone formation in intermaxillary suture while LMWHA had no effect(42). These results were conflicted by the outcomes of our study, which could be attributed to the different region of interest for force application in Sadikoglu study. In addition, the space produced between two maxillary halves by expansion spring may lead to other cascades of biological events.

In term of bone volume density results, the decrease in BV/TV ratio means increase in the osteoclastic activity and decrease in the resistance of the bone to the OTM (9). In our study, the bone volume density were decreased significantly after day 14 of the experiment and continue until day 21 in both groups of HA injection which may induced acceleration of orthodontic treatment. These bone density related outcomes lead us to think that possible increase in osteoclasts number in the compression side lead to decrease the bone volume density in the interradicular area of involved molar. The calculation of osteoclasts number was used in most of the studies on acceleration of OTM (148,153,256,286). According to our findings the osteoclasts number at experimental side was increased significantly on day 14 and day 21 in comparison to control side, which corresponds to the days on which bone density was decreased.

Because of the proved relation of RANKL and OPG to the bone remodelling process, the level of expression of these proteins by immnuhistochemical analysis were evaluated in many studies of OTM (286–288), both the RANKL and OPG expression was found to be significantly increased in the experimental side more than the control side especially on day 21. The biological function of OPG in the alveolar bone remodelling is the inhibition of osteoclast function and the acceleration of osteoclast apoptosis (289). Increased amount of OPG in bone could suggest that the attempt to of accelerate the tooth movement in our study was conflicted by normal physiological response of bone to maintain adequate bone remodelling rise in the RANKL level (290).The ratio of expression of RANKL in the bone tissue to that of OPG was better

indicative of the bone remodelling process, RANKL/OPG ratio was significantly higher in experimental side than control side on day 14 and day 21, which means that RANKL had increased in a rate more than OPG, which means that the bone remodelling balance inclined toward osteclastogensis rather than bone formation.

ALP is another bone marker evaluated in studies of OTM to elucidate the activity of osteoblasts (291,292). ALP expression was significantly higher in experimental sides than control sides on day 14 and with fall down on day 21, this could be as a result of hyalinization process occurred during OTM which peaked on day 14 and reduced after that (293).

The role of OCN in bone formation had been illustrated in previous literature (294), because of its negative regulation of bone mineralization and formation, it was considered as a bone marker during tooth movement. Hashimoto et al. revealed in his study that local injection of exogenous OCN accelerated OTM significantly (141), in our study, the expression of OCN showed no significant difference between experimental and control side. However, the values of OCN expression were increased significantly between day 3 and day 21 in experimental sides.

Both catabolic and anabolic activities of the bone are the determining factors in the amount of OTM, but the amount of bone resorption in the direction of movement is key factor in the acceleration of tooth movement (61). The OTM begin as 1st phase tooth movement inside the socket followed by bone resorption and apposition in the 2nd phase. Frost et al elucidated that bone response to external stimuli lead to increase in the rate of local remodelling to repair the bone in the shortest time, this phenomena was called regional accelerating phenomena (73). The application of OTM combined with HA injection could act like a stimulus for the previously mentioned frost phenomena.

CONCLUSION:

- High (6000 kDa) and low (500 to 750 kDa.) molecular weight HA injection resulted in an increase in rate of OTM through out all the experimental days.
- The amounts of tooth movement in the experimental side of HMWHA group were 1.4 times greater than control side and 1.2 times greater than the experimental side of LMWHA group.
- Both high and low molecular weight HA injection resulted in decrease in the bone density between the dental roots.
- The RANKL/OPG pathway tends to show more Osteoclastogenesis in groups injected with HA. With RANKL increasing in a rate greater than OPG.
- Hyaluronic acid can be considered as a good stimulus for acceleration of orthodontic tooth movement.

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7. APPENDICES

7.1 Ethical Approval

T.C.
GAZİANTEP ÜNİVERSİTESİ
UNIVERSITY OF GAZIANTEP
HAYVAN DENEYLERİ YEREL ETİK KURULU BAŞKANLIĞI
LOCAL ETHICS COMMITTEE OF ANIMAL EXPERIMENTS
ARAŞTIRMA BAŞVURU ONAYI
RESEARCH APPLICATION CONSENT

TOPLANTI TARİHİ (Meeting Date)	**TOPLANTI SAYISI** (Meeting No)	**TOPLANTI YERİ** (Meeting Place)
08.01.2019	1	Hayvan Deneyleri Yerel Etik Kurulu

BAŞVURU BİLGİLERİ Application Information	Araştırmanın Başlığı Research Title	Hyalüronik asidin ortodontik diş hareketi esnasında alveoler kemik yeniden şekillenmesi (remodelling) üzerine etkisi The effect of hyaluronic acid on the remodelling of alveolar bone during orthodontic tooth movement
	Başvuru Tarihi Application Date	02.01.2019
	Protokol no Protocol no	82

KARAR BİLGİLERİ Decision	**Karar No:** 2019/3 **Decision No:** 2019/3 ☒ Kabul (Accepted) ☐ Red (Not Accepted) Dr. Öğr. Üyesi Merve GÖYMEN'in yürütücüsü olduğu ve yukarıda başvuru bilgileri verilen araştırma başvuru dosyası ve ilgili belgeler araştırmanın gerekçe amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş, başvurunun "uygun" olduğuna toplantıya katılan üyelerin oy birliği ile karar verilmiştir.

Yürütücü Coordinator	Dr. Öğr. Üyesi Merve GÖYMEN Gaziantep Üniversitesi, Diş Hekimliği Fakültesi, Ortodonti Anabilim Dalı

ETİK KURUL BİLGİLERİ	
ÇALIŞMA ESASI	GAZİANTEP ÜNİVERSİTESİ HAYVAN DENEYLERİ YEREL ETİK KURULU YÖNERGESİ

Ünvanı / Adı / Soyadı	Kurumu	İlişki	Katılım	İmza
Prof. Dr. A. Tuncay DEMİRYÜREK (Başkan)	GAÜN Tıp Fakültesi Tıbbi Farmakoloji AD.	H	E	
Prof. Dr. Behçet AL (Üye)	GAÜN Tıp Fakültesi Acil Tıp AD.	H	E	
Prof. Dr. Tekin KARSLIĞİL (Üye)	GAÜN Tıp Fakültesi Tıbbi Mikrobiyoloji AD.	H	H	KATILMADI
Prof. Dr. Sibel OĞUZKAN BALCI (Üye)	GAÜN Tıp Fakültesi Tıbbi Biyoloji AD.	H	E	
Prof. Dr. Emine Elçin EMRE (Üye)	GAÜN Fen Edebiyat Fakültesi Kimya Bölümü	H	E	
Doç. Dr. Can DEMİREL (Üye)	GAÜN Tıp Fakültesi Biyofizik AD.	H	E	
Dr. Öğr. Üyesi Davut Sinan KAPLAN (Üye)	GAÜN Tıp Fakültesi Fizyoloji AD.	H	E	
Dr. Öğr. Üyesi Merve GÖYMEN (Üye)	GAÜN Diş Hekimliği Fakültesi Ortodonti AD.	E	E	
Öğr. Gör. Dr. Ahmet Sarper BOZKURT (Üye)	GAÜN Teknik Bilimler MYO Bitkisel ve Hayvansal Üretim	H	E	
Veteriner Hekim Celal ÖZSÖYLER (Üye)	Gaziantep Büyükşehir Belediyesi Hayvanat Bahçesi	H	E	
Avukat Orhan BEYAZ (Üye)	Serbest, Dernek Üyesi	H	E	

E: Evet, H: Hayır

8. CURRICULUM VITAE

Adı Soyadı: Dler MOURAD

Doğum Tarihi: 15/01/1985

Öğrenim Durumu: Doktora Öğrencisi

Derece	Bölüm/Program	Üniversite	Yıl
Lisans	Diş hekimliği	Hawler Medikal Üniversitesi	2004-2009
Yüksek Lisans	Ortodonti	Gaziantep Üniversitesi	2012-2014

Yüksek Lisans Tezi Başlığı ve Danışmanları: Ortodontik Metal Braketlerin Farklı Yüzey Pürüzlendirme Yöntemleri Uygulanan Amalgam Yüzeylerine Olan Bağlanma Dayanıklılığının Değerlendirilmesi. Yrd.Doç.Dr. N.Eren İŞMAN (Gaziantep Üniversitesi)

Görevler:

Görev Ünvanı	Görev Yeri	Yıl
Yüksek Lisans Öğrencisi	Gaziantep Üniversitesi Diş Hekimliği Fakültesi	2012-2014
Doktora Öğrencisi	Gaziantep Üniversitesi Diş Hekimliği Fakültesi	2014-2020

Bilimsel Kuruluşlara Üyelikler :

1- Türk Ortodonti Derneği (TOD)

Yabancı Dil:

TOEFL-IBT (Test of English as a Foreign Language-Internet Based Testing) : 85.00

ESERLER

A. Uluslararası hakemli dergilerde yayımlanan makaleler

1. Göymen M, **Mourad D**, Güleç A. Evaluation of Airway Measurements in Class II Patients Following Functional Treatment. Turkish Journal of Orthodontics. 2019 Mar;32(1):6.

B. Uluslararası bilimsel toplantılarda sunulan ve bildiri kitabında (*Proceedings*) basılan bildiriler :

1. In-Vitro Assessment of Shear Bonding Strength of Metal Brackets to Amalgam Restorations Using Different Surface Conditioning Methods. **Mourad D**, Işman N. 14. International Congress of the Turkish Orthodontic Society, October 25th – 29th 2014, Ankara, Turkey.

2. Effects Of Using Plaque Disclosing Tablets At Home On Oral Hygiene In Orthodontic Patients. Yavan M; Sökücü O; **Mourad D**; Göymen M; Özdemir S. 20th Congress of the Balkan Stomatological Society, April 23rd – 26th 2015, Bucharest, Romania.