



T.C.
YEDITEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF ORTHODONTICS

HISTOMORPHOMETRIC AND BIOCHEMICAL EVALUATION OF THE EFFECTS OF DIFFERENT MOLECULAR WEIGHT HYALURONIC ACID ON BONE FORMATION IN RATS

DOCTOR OF PHILOSOPHY THESIS

ALI GÖRDEAL, DDS

Istanbul- 2022



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This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated and numbered

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

07.04.2022

Ali Gördeal



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TABLE OF CONTENTS

APPROVAL FORM	ii
DECLARATION	iii
ACKNOWLEDGEMENTS	iv
TABLE of CONTENTS	v
LIST OF TABLES	vii
LIST OF FIGURES	vii
LIST OF SYMBOLS AND ABBREVIATIONS	ix
ABSTRACT	xi
ABSTRACT (Turkish)	xiii
1. INTRODUCTION and PURPOSE	1
2. LITERATURE REVIEW	2
2.1. Occlusion and Malocclusion	2
2.2. Transversal Anomalies	3
2.3. Etiology of Maxillary Deficiency	4
2.4. History of Treatment of Maxillary Deficiencies and Current Approaches	5
2.5. Indications, Timing and Retention of the Rapid Maxillary Expansion	7
2.6. Craniofacial Structures Affected by Rapid Maxillary Expansion	9
2.7. Bone Tissue	11
2.7.1. Bone Structure Cells	13
2.7.1.1. Osteoprogenitor Cells	13
2.7.1.2. Osteoblast Cells	14
2.7.1.3. Osteocyt Cells	14
2.7.1.4. Osteoclast Cells	15
2.8. Osteogenesis	15
2.8.1. Intramembranous Ossification	16
2.8.2. Endochondral Ossification	16
2.8.3. Ossification in Dentofacial Sutures	17
2.8.4. Healing of Fractured Bone Structure	18
2.8.5. Bone Remodelling Metabolism	19
2.9. Structure of the Maxilla and Midpalatal Suture	19
2.10. Hyaluronic Acid	20

2.10.1. Chemical Structure of Hyaluronic Acid and Level of Hyaluronic Acid in Body	21
2.10.2. High and Low Molecular Weight Hyaluronic Acid	22
2.10.3. Local and Systemic Effects of Hyaluronic Acid	24
2.10.4. Hyaluronic Acid in Human and Animal Studies	26
2.11. Use of Rats as Experimental Animals	30
2.11.1. Dentofacial Structure and Anatomy of Rats	31
2.11.2. Bone Healing Metabolism of Rats	32
3. MATERIALS AND METHODS	33
3.1. Animals and Groups	33
3.1.1. End Point Criterias of Experimental Animals for This Study	35
3.2. Materials Used in The Experiment	36
3.2.1. Materials Used in Histological and Biochemical Investigation	37
3.3. Method	38
3.3.1. Histological and Histomorphometrical Evaluation	40
3.3.2. Blood Samples and Obtaining Blood Serum Plasma	46
3.3.2.1. Biochemical Analysis	47
3.4. Statistical Evaluation	49
4. RESULTS	50
4.1. Observational Results	50
4.2. Histomorphometrical Findings	52
4.2.1. Bone Volume	52
4.2.1.1. Comparison of 10 and 20 Days Experiment Groups Separately	53
4.2.2. Soft Tissue Volume	56
4.3. Biochemical Results	59
5. DISCUSSION	60
6. CONCLUSION	72
7. REFERENCES	73
8. APPENDICES	91
8.1. Ethical Approval	91
9. CURRICULUM VITAE (Turkish)	92

LIST OF TABLES

Table 1. Mean and standart deviation of weight of rats in day 0, 5, 10 and 20	33
Table 2. Number of animals for each experimental group	35
Table 3. Tissue Fixation Stages	42
Table 4. Stages of Masson's trichrome staining technique	43
Table 5. Weight loss in control and experiment group animals	50
Table 6. Bone volume levels and comparison of all groups	52
Table 7. Comparison of bone volume of 10 days experiment groups	53
Table 8. Comparison of bone volumes of 20 days experimental groups	55
Table 9. Soft tissue volume levels and comparison of all groups	56
Table 10. Average blood plasma HA level and standart deviation of all groups	59

LIST OF FIGURES

Fig 3.1. Polycarbonate cages, identificatin numbers on cages	34
Fig 3.2. Marking their ears and tails for identification	34
Fig 3.3. Drilling Hole and Preperation for Helix Spring	38
Fig 3.4. Application of Helix Spring	39
Fig. 3.5. Measurement of Expansion amount after 5 days Expanison and Retantion	39
Fig. 3.6. High Molecular Weight Hyaluronic Acid (Healon GV®)	40
Fig. 3.7. Low Molecular Weight Hyaluronic Acid (AMO Vitrax II®)	40
Fig. 3.8. Salin Solution	40
Fig. 3.9. Dissected rat maxilla	41
Fig. 3.10. Paraffin blocks were sectioned longitudinally (10 mm thick) parallel to the long axis of each tooth, from mesial to distal aspects	41
Fig. 3.11. Stereological work station	44
Fig. 3.12. Midpalatal suture area point counting	45
Fig. 3.13. Obtaining blood serum plasma	46
Fig. 3.14. Blood serim hyaluronic acid level indicator set (Shanghai Yehua Biological Technology, Shanghai, China)	47
Fig. 3.15. Blood serum hyaluronic acid level indicator set application procedure	48
Fig. 4.1. Weight loss comparisons on days 5, 10 and 20 among the groups	51
Fig. 4.2. Bone volume levels for all groups	53
Fig. 4.3. Bone volume levels of 10 days group	54
Fig. 4.4. Bone volume levels of 20 days group	55
Fig. 4.5. Soft tissue volume levels for all groups	57
Fig. 4.6. Soft tissue volume levels of 10 days group	58
Fig. 4.7. Soft tissue volume levels of 20 days group	58
Fig. 4.8. Blood plasma serum HA levels for all groups	59

LIST OF SYMBOLS AND ABBREVIATIONS

CC	Cubic Centimetres
CT	Computed Tomography
CBCT	Cone Beam Computed Tomography
CPS	Character Per Second
Da	Dalton
ECM	Extra Cellular Matrix
EHA	Esterified Hyaluronic Acid
GAG	Glucose Aminoglycan
Gr	Grams
HA	Hyaluronic Acid
HxE	Hematoxylin Eosin
HMW	High Molecular Weight
IgG	Immune Globulin G
Kg	Kilogram
LMW	Low Molecular Weight
MA	Molecular Weight
Mg	Milligram
Min	Minute
ml	Milliliter
mm	Millimeter
MDa	Million Dalton (Molecular Weight Unit)
Mpas	Millipascal second (Unit of Viscosity)
NaHA	Sodium Hyaluronate
Nm	Nanometer
N	Newton
OA	Osteoarthritis
OH:	Hydroxyl
OVD	Ophthalmic Viscous Surgical Device
PDL	Periodontal Ligament
PDGF	Platelet Derived Growth Factor

PGE ₂	Prostaglandin E ₂
PRP	Platelet Rich Plasma
PRF	Platelet Rich Fibrin
RME	Rapid Maxillary Expansion
RANKL	Receptor Activator of Nuclear Factor
RHAMM	Hyaluronan Mediated Modality Receptor
RNA	Ribonucleic acid
SD	Standard deviation
TGF	Transforming Growth Factor
μm	Micrometer



ABSTRACT

Gördeal, A. (2022). Histomorphometric and Biochemical Evaluation of the Effects of Different Molecular Weight Hyaluronic Acid on Bone Formation in Rats. Yeditepe University, Institute of Health Sciences, Department of Orthodontics, PhD thesis, İstanbul.

The aim of this study was to evaluate the histomorphometric and biochemical effect of locally applied hyaluronic acid solution with different molecular weight on bone formation in the long and short term, injection applied into the midpalatal suture area in rats. This study was conducted on 48 male Sprague-Dawley rats with an average weight of $360,31 \pm 52,2$ gr. The rats were divided into 3 groups; control (saline application only), LMWHA (low molecular weight hyaluronic acid), HMWHA (high molecular weight hyaluronic acid). Two different time intervals were examined for each group: 10th and 20th days groups after injection. In all groups, rats maxillas were expanded with stainless steel helix expansion spring. On the 5th day, helix spring was removed and replaced with retention wire. Simultaneously with retention procedure, 0,05 ml (50 μ l) high and low molecular weight hyaluronic acid and saline solution was locally injected to midpalatal suture area. After the 10th and 20th day of injection rats were sacrificed. Blood samples were collected and upper jaw samples were dissected and prepared for histological examination. For histomorphometric examination, dissected and trimmed blocks were longitudinally sectioned serially, 10 μ m thickness intervals were applied parallel to long axis of the incisors. The sections were stained with Masson's trichrome were analyzed by photo microscope. Bone and connective tissue volume measurements were made. Blood serum plasma were obtained from blood samples by centrifugation, and the amount of HA (hyaluronic acid) in the serum was determined using HA measurements kits. Bone formation amount showed a significant difference between the control and experimental groups on the 10th and 20th days ($p < 0,05$). Bone area (μm^2), and bone perimeter (μm) and blood serum HA values of each group were examined. As a result of histomorphometric examination, high molecular weight hyaluronic acid solution significantly increased bone formation in the long and short term compared to low molecular weight hyaluronic acid and control groups ($p < 0,05$). Also low molecular weight HA groups showed significantly high bone formation values according to control

groups ($p < 0,05$). When blood serum HA levels were examined, LMWHA groups showed high values but there was no significant difference between the blood serum HA values of experimental and control groups ($p > 0,05$). In this study, it was aimed to accelerate bone formation, shorten orthodontic retention time and decrease relapse with a minimally invasive approach, and also aimed to elucidate the systemic spread characteristics of locally applied HA solutions by analyzing to blood serum HA data biochemically. Bone formation is increased by local application of high and low molecular weight hyaluronic acid. This study shows that, local injection of HMWHA and LMWHA solution in midpalatal suture area after rapid maxillary expansion stimulated new bone formation that may shorten retention time and may reduce the risk of relapse.

Key words: Rapid maxillary expansion, hyaluronic acid, accelerated bone formation, orthodontic relapse, blood plasma, rats

ÖZET

Gördeal, A. (2022). Farklı Molekül Ağırlıklarında Hyalüronik Asit Preparatlarının Kemik Oluşumuna Etkisinin Sıçanlarda Histomorfometrik Ve Biyokimyasal Olarak İncelenmesi. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Ortodonti ABD., Doktora Tezi. İstanbul.

Çalışmamızın amacı, hızlı üst çene genişletmesi uygulanan sıçanların midpalatal sutur alanlarına enjekte edilen farklı moleküler ağırlığa sahip hyalüronik asidin uzun ve kısa dönemde kemik oluşumuna olan etkisinin histomorfometrik ve biyokimyasal olarak incelenmesidir. Bu çalışma ortalama ağırlıkları 360,31 $\pm$ 52,2 gr olan 48 adet erkek Sprague-Dawley sıçanı üzerinde yürütülmüştür. Sıçanlar 3 gruba ayrılmıştır: kontrol (sadece serum fizyolojik), DMAHA (düşük moleküler ağırlıklı hyaluronik asit) ve YMAHA (yüksek moleküler ağırlıklı hyalüronik asit). Her grup için iki farklı zaman aralığı incelenmiştir: 10. ve 20. günler. Tüm gruplarda sıçanların üst çenesi paslanmaz çelik heliksli genişletme apareyi yardımıyla genişletilmiş ve 5. günde aparey çıkartılarak retansiyon apareyi uygulanmıştır. Retansiyon işlemiyle eşzamanlı olacak biçimde yüksek ve düşük moleküler ağırlıklı hyaluronik asit ile serum fizyolojik solüsyonu midpalatal sutur alanına bölgesel olarak 0.05 ml (50 μ l) enjekte edilmiştir. Sıçanlar takip gruplarına göre 10. ve 20. günlerde sakrifiye edilmiştir. Kan örnekleri toplanmış ve üst çene örnekleri diseke edilip histolojik incelemeye hazırlanmıştır. Histomorfometrik inceleme için kesici dişlerin uzun eksenine paralel olacak biçimde, dişlerin kesici kenarlarından apeksine doğru 10 μ m kalınlığında uzunlamasına seri kesitler alınmıştır. Masson's trichrome ile boyanan kesitler foto mikroskobu ile analiz edilmiş ve hacim ölçümleri yapılmıştır. Alınan kan örnekleri santrifüj edilerek kan serumları elde edilmiş ve HA ölçüm kitleri kullanılarak serum içerisindeki HA miktarları belirlenmiştir. Kemik oluşum miktarları 10. ve 20. günlerde kontrol ve deney grupları arasında anlamlı bir farklılık göstermiştir ($p<0.05$). Her grubun kemik alanı (μm^2) ve kemik perimetresi (μm) ve kan serum HA değerleri incelenmiştir. Histomorfometrik inceleme sonucunda yüksek moleküler ağırlıklı hyaluronik asit solüsyonunun düşük moleküler ağırlıklı hyaluronik asit solüsyonu ve kontrol grubuna göre uzun ve kısa dönemde kemik yapımını anlamlı derecede arttırdığı gözlenmiştir ($p<0.05$). Düşük moleküler ağırlıklı HA solüsyonu ve kontrol grubu karşılaştırıldığında DMAHA grupları kontrol gruplarına göre anlamlı derecede kemik

üretimi arttırdığı gözlenmiştir ($p<0.05$). Kan serumları HA değerleri incelendiğinde DMAHA 10 ve 20 gün grupları yüksek değerler gösterse de anlamlı bir fark bulunamamıştır ($p>0.05$). Bu çalışmada minimal invaziv yaklaşımla kemik oluşumunun hızlandırılması ve ortodontik retansiyon süresinin kısaltılması, nüksün azaltılması amaçlanmıştır ayrıca kan serumu HA verilerinin biokimyasal olarak incelenerek bölgesel uygun HA solüsyonlarının sistemik yayılım karakterlerinin aydınlatılması amaçlanmıştır. Bölgesel olarak yüksek molekül ağırlıklı hyaluronik asit uygulamasıyla kemik oluşumu artırılmıştır. Bu çalışma HA kullanımı sayesinde ortodontik tedavilerde retansiyon süresinin kısaltılabileceği ve nüks riskinin azaltılabileceğini göstermektedir.

Anahtar kelimeler: Hızlı üst çene genişletme, hyaluronik asit, hızlandırılmış kemik oluşumu, ortodontik nüks, kan serumu, sıçan

1.INTRODUCTION AND PURPOSE

In present moment, orthodontic treatments aim to achieve healthy, functional and aesthetic results and target to ensure the continuity of these gains and prevent their relapse.

Following the end of orthodontic treatment, the teeth and skeletal structure tend to return to the pretreatment positions and this condition is called relapse. It is affected by growth and development changes, occlusal and periodontal factors, soft and hard tissue tension and pressure (1).

It is observed that the upper jaw bone gets narrow again in patients who do not use retention protocol, following the rapid maxillary expansion applications, which is a frequently preferred treatment method for transversal malocclusions in orthodontics. This return can reach up to 90% (2). This negatively affects the patient's motivation to treatment.

There are many animal and human studies aiming to increase bone formation, decrease relapse and reduce retention time after rapid palatal expansion (3,4,5). In this context, many agents are applied locally to the suture area. Although many substance applications have been tried in the suture field, there is no any HA application with long and short term observations. Also, there are limited studies examining the levels of administered agents in blood serum and the effects of local applications on peripheral tissues.

In our study, it was planned to compare the effect of high and low molecular weight hyaluronic acid (HA) locally applied to the upper jaw midpalatal suture area on bone formation after rapid palatal expansion and to examine the amount of hyaluronic acid in the blood serum in different time intervals.

The aim of this study is evaluate the histomorphometric effects of different molecular weight on bone formations for long and short term. Also, biochemical observation to understand mechanism of hyaluronic acid physiologic mechanism.

2.LITERATURE REVIEW

2.1. Occlusion and Malocclusion

The concept of “normal” or “ideal” occlusion has been tried to define from many point of views to the present day. Edward H. Angle, the founder of modern orthodontics, made the classification of ideal occlusion and malocclusions in the late 18th century. According to this definition, in the normal occlusion, the lower first molar occludes a distance of half the mesio-distal width of a premolar mesially than the upper first molar. Transversally, the upper teeth overlap the lower teeth (6,7,8)

In the definition of ideal occlusion, not only teeth but also lower and upper jaw bones are considered to be in an ideal position relative to each other. The relationship of the lower jaw on the horizontal plane relative to the upper jaw is explained by the following three positions; centric relationship, protrusiv relationship, lateral relationship. Among these, centric relationship is the most discussed topics of dentistry (9).

According to the Webster dictionary, the word “centric” comes from the Greek word “kentrikos”, which means “center”. Centric relationship is defined as the maxillomandibular relationship in which the condyles are associated with the thinnest and undamaged parts of their own discs and the condyle disc-complex is in the anterior-superior position against the articular eminence (9).

In the position where this relationship is achieved, the teeth are in the “ideal occlusion”, in the case of the maximum intercuspitation. The ideal occlusion is difficult to obtain and not found in every person. “Normal occlusion”, “neutral occlusion”, “ideal occlusion” definitions are all synonymous. The presence of deviations occurring outside the definitions is called malocclusion (6).

The occurrence of malocclusion is not due to a single cause. The lower and upper jaw bones, teeth, other facial bones, the chewing and mimic muscles that form the neuromuscular system, and the sensory and motor nerves that feed them; etiological factors such as hereditary factors and environmental factors, trauma, bad habits, malnutrition, diseases are effective (10, 11). These factors can be effective pre- and

postnatal. The age of the patient and duration of action are factors that determine the severity of malocclusion (12,13).

Malocclusions are classified under two general types: intramaxillary and intermaxillary. Intramaxillary anomalies have many types such as crowding, spacing, rotations, diastemas, vestibuloposition, lingoposition, infraposition, supraposition, mesioposition, and disposition of the teeth. Intermaxillary anomalies are classified under three main types: sagittal, transversal and vertical anomalies. Constriction is a transverse direction anomaly (7, 10).

2.2. Transversal Anomalies

A malocclusion is called “crossbite” when the upper posterior teeth are positioned more palatinally than their antagonists (10). Transverse direction anomalies are generally caused by the inadequate width of the upper jaw relative to the lower jaw and there are several factors that are effective in its formation (14). If the transverse direction anomaly is observed in the posterior teeth, it is called posterior crossbite (15). The cause of posterior crossbite may be dental or skeletal. Even if the palatal width is adequate, posterior crossbite can be seen due to dental anomalies (16).

The dental posterior crossbite is characterized by palatal tipping of the posterior teeth when the arch width is normal. This may occur for a single tooth or a group of teeth. The dental posterior crossbite is often caused by local factors (17).

In the skeletal posterior crossbite, the transverse direction anomaly between the maxilla and the mandibula is mostly due to the bilateral or unilateral narrowing of the maxilla. The apical bone base is insufficient in maxilla. Posterior crossbite may also occur with the normal development of the maxilla and excessive development of the mandible (17-19).

Transversal anomalies are divided into groups such as palatal nonocclusion, unilateral and bilateral crossbite, and buccal nonocclusion. Treatment methods and approaches may vary depending on the type of anomaly (10).

2.3 Etiology of Maxillary Deficiency

Factors such as heredity, prolonged use of pacifier or bottle and pathological mouth breathing lead to maxillary deficiency. Untreated maxillary deficiencies cause TMJ disorders and asymmetry development in the face (20).

Finger sucking during primary dentition period may lead to the formation of unilateral crossbite (21). Increased muscular activity on the anomaly side as a result of maxillary deficiency affects craniofacial growth and leads to facial aesthetics deterioration along with the development of asymmetry.

The individual is programmed to perform nasal breathing following birth. During nasal breathing, the tongue takes a position to support the palate, thereby neutralizing the constrictive effect of chewing and facial muscles on the upper jaw (17, 22). Nasal obstruction is frequently seen in the maxillary deficiency. With the orthodontic treatments performed in the early period, the patients can breathe through the nose again and the nasal obstruction problem can be corrected. (23) As a result of narrowing of the maxilla in cases with no orthodontic treatment in the early period, the mandible rotates clockwise and as a result of this increase in the facial size, the patients develop adenoid face type. Maxillary deficiency has been observed in rhesus monkeys with experimental nasal obstruction (24).

Apart from the nasal obstruction, the cleft lip and palate and craniosynostoses may be the cause of the skeletal problem. Synostosis is defined as the closure of sutures in the early period. Although cranial synostosis is more common, early closure of sutures seen in the facial bones is rare. As Kokich points out, the zygomatic-temporal suture is one of the late closing sutures and is very effective in the growth period. (25)

According to functional matrix theory, growth occurs in response to functional needs. Soft tissues grow and bone and cartilages respond to this growth (26). Correct functioning of the orofacial region (such as breathing, chewing and swallowing functions) causes correct growth and development of the jaw bones (27, 28).

Chierici et al. (29) formed 3 mm wide scars on the hard palate of apes. They left the soft palate and nasal cavity mucosa unharmed and examined the response of a normal structure to abnormal environmental conditions. They observed a crossbite due to maxillary, nasal cavity and tooth position changes.

2.4. History of the Treatment of Maxillary Deficiencies and Current Approaches

In his article published in 1860 by Emerson C. Angell, the investigator suggested that midpalatal suture could be opened by means of a bi-directional screw between the premolars. Angell applied his appliance to a 14-year-old girl. He told the patient to twist the screw twice a day. Angell reported that the upper jaw was expanding too much after 2 weeks of expansion and that the space between the upper incisors indicated that the maxillary bones were separated from each other. The crossbite of the left upper incisor tooth was corrected. Since X-ray was not present at the time, his views were highly criticized and skeptical (30).

This idea was then reported by Farrar (1888), by Goddard (1893), by G.V. Black (1893) and Monson (1898) but it did not gain much popularity over the next fifty years. The acceptance of the functional concept in growth and development had been effective in that period of time (30).

Although Brown (1903) stated that the nasal septum and nasal passages were affected by upper jaw expansion, he could not support this view radiologically due to technical deficiencies. Landsberger (1910) was able to show the expansion of the midpalatal suture as a result of lateral forces applied to the posterior teeth for the first time on occlusal radiography. Although various researchers used this technique from 1911 to 1929, the rapid maxillary expansion did not gain much popularity until 1961 (31).

Derichsweiler showed that the two maxillary bones were separated from each other as a result of expansion with the frontal radiographs obtained before and after treatment (31).

In 1940, Graber began using the rapid maxillary expansion device in the treatment of patients with cleft lip and palate (32).

In 1961, Haas introduced a fixed, tissue-supported appliance named after him. The device consists of 4 bands of upper 1st premolars and molars and thick wires soldered to the buccal and lingual surfaces of each band. The lingual wires extend towards the palatal side and are embedded in the acrylic plate, the screw is placed along the middle palatine suture so that the lateral edges of the expansion screw are contained within the acrylic. According to the investigator, because of the acrylic plate of the device, there is less tipping movement of the teeth. The force is applied not only on the teeth, but also on the soft and hard tissues of maxilla, which are covered by the appliance. As a result, the bodily movement in the maxilla can occur (19, 33).

In 1960, Ricketts expanded the upper jaw with a device called Quad-helix by banding the upper permanent first molars and soldering 4-helix W-shaped wire made of 1 mm round steel wire. It is stated that this device transmits light, continuous forces and has skeletal effects especially when applied in primary and mixed dentitions. It is reported that the device is more advantageous in patients with cleft lip and palate, as more movement can be achieved in the anterior region than the posterior region (34, 35).

Wertz (36), in his study, found that maxillary expansion increases nasal air flow in patients with nasal stenosis. In their work in the 1970s, Wertz (37), Melsen (38), Persson and Thilander (39) questioned the relationship between skeletal maturation and effective sutural opening.

Biederman and Chem published a study describing the treatment of class III cases with maxillary expansion and introduced the Hyrax device, which is more hygienic in this respect, which does not carry an acrylic base as in the Haas appliance (40, 41).

Subtelny (42) reported that in the high angle cases tooth crowns were better grasped by bite plate attached expanders, less tipping occurred in the molar teeth, and that this device delivered more force to the nasomaxillary complex through the tooth roots.

Timms (43) introduced the Cap Splint apparatus consisting of two separate chrome cobalt plates and one screw covering the occlusal surfaces of all teeth except the upper central teeth.

Howe (44) presented a case in which he treated with the apparatus he referred to as an acrylic bondable palatal expansion device.

Arndt (45) developed the “nickel titanium expansion device” to provide expansion in the maxillary arch. The investigator reported that this device provides equal palatal expansion in the transversal direction in the anterior and posterior region, and also provides ease of application for the patient as well as ease of application for the orthodontist.

Darendeliler et al. (46), expanded the maxilla with samarium cobalt magnets, applying 250-500 gr continuous force. Researchers have reported that more permanent results can be obtained with light but continuous forces.

In 1999, a transpalatal distractor device was applied completely in bone tissue and the disadvantages such as root resorption, fenestration, recurrence during or after expansion, elongation in teeth and tipping were eliminated (47).

Toroğlu et al. (48) reported that they had expanded the crossbite side of the maxilla with the AMEX device they applied in cases of true unilateral maxillary deficiencies and this device was effective in the treatment of unilateral posterior crossbites.

2.5. Indications, Timing and Retention of the Rapid Maxillary Expansion

General indications for rapid maxillary expansion are as follows;

- Unilateral and bilateral crossbites (19),
- In order to increase the arch length and width in crowding cases where there is no indication of dental extraction (49),
- In Angle Class II div. 1 malocclusions, in order to provide the mandible to come forward spontaneously (50),
- To reduce nasal resistance to assist breathing (49,50),
- In order to expand the collapsed maxilla in cases of cleft lip and palate (49,50),
- In order to ensure that cephalometric point A comes forward in the treatment of mild anterior crossbite cases (19, 51, 52).

According to Izzard's study on the skulls; midpalatal suture begins to close around age 16 and is completely ossified around age 25. Therefore, it has been reported that slow maxillary expansion should be performed in the 7-12 age range. (53)

As the patient's age increases, the resistance in the suture increases also and the force required for skeletal expansion increases (54-56).

In the studies, it was found that total ossification age of midpalatal suture varies according to personal factors and gender. The suture could close in a 15-year-old girl; however, a 27-year-old woman could still have not completed the ossification. It has been reported that midpalatal suture can be separated in adult individuals up to 25 years of age (39).

Da Silva et al. (57) argue that expansion treatment should be performed as early as possible, including during the primary dentition period because this correlates with the easier expansion of the maxilla and the reduction in pain symptoms of patients.

McNamara reported that the results of rapid maxillary expansion were more predictable in early permanent dentition in young patients. The reason for this is reported to be the suture structure in young individuals (58).

In the studies comparing the effects of rapid maxillary expansion according to age, it is argued that orthopedic effect occurring at early ages remains below the expected, and that it may be a better alternative to wait until early mixed dentition period (59).

Björk (60) stated that according to radiological findings, at the age of 17 sutural development is over.

In rapid maxillary expansion cases; the supportive tissue (33), retention time (61), age of the patient (62), retention method (63) and surrounding soft tissues (33, 37, 64-67) have an effect on the relapse.

After rapid maxillary expansion, sufficient retention time is needed for stable results (68,69). The researchers have different opinions about the retention time.

Some studies (14,69,70,71) report that the changes in the midpalatal suture after expansion can be seen radiologically 3 months later. Histologically, 6 months later the calcification at the suture area is completed. It was also reported that calcification of the

entire suture from the anterior nasal spina to the posterior nasal spina lasted for 8-9 months (72).

There are studies showing that the amount of excessive forces after rapid expansion in maxillary and craniofacial structures is a cause of relapse. In some studies, it is reported that the cause of relapse is the tension of soft tissues around skeletal structures (70, 73, 74).

Storey (75) stated that a more biological adaptation was formed in the midpalatal suture with slow expansion, and sutural stability was better. It was reported that there was less tipping in the supported teeth with slow expansion and less deposition of residual forces in the expanded segments. Because of the protection of the sutural integrity and the accumulation of less force in the tissues, the clinicians thought that the relapse would be less with slow expansion (54, 75, 76). Sutural regeneration and stabilization are achieved with 3-month or less retention of maxillary segments separated by slow widening (63,69,71,75)

Relapse after rapid expansion is related with the type of the retention appliance. Relapse was observed in 45% of the patients who did not use any retention appliance, while this rate decreased to 22-25% in patients using removable appliances such as Hawley, wraparound. However, as retention appliances require patient compliance, relapse rates are reduced to 10-23% in patients with fixed retainers (73).

2.6 Craniofacial Structures Affected by Rapid Maxillary Expansion

In many rapid maxillary expansion methods, the expansion force is transmitted to the tissues by means of a screw placed in the midpalatal suture region. In each activation of the screw a lateral force occurs. Activations of the same amounts form varying amounts of forces in different patients and in the same patient at different stages of treatment (77).

Orthopedic force caused by activation of the screw causes the opening of the midpalatal suture. The appliance makes pressure on the periodontal ligament and the alveolar bone and teeth are slightly bent (33). The maxilla is displaced downward and

forward in the horizontal and frontal planes. The opening in the midpalatal suture can be easily detected by occlusal radiographs. The maxillary fragments are separated from the midpalatal suture to form a radiolucent triangular area in the anterior, where the resistance of the facial structures is less (19, 33, 37, 69, 78).

When occlusal films were examined, it was observed that the opening in the suture extended to the horizontal plates of the palatine bones (37). From the frontal view, it is observed that the maxillary suture is not fragmented parallelly in the superoinferior direction. The opening consists of a pyramidal structure. The base of the pyramid is in the roof of the mouth and the rotation center in the frontonazal suture.

According to Newton's action reaction law, resistance will also occur in the affected structures when the expansion screw is turned. This resistance depends on the age of the patient, the bone density in the sutures of the maxilla, especially the midpalatal, maxillo-zygomatic, maxillopalatine and sphenopalatine sutures, the degree of ossification and the stages of treatment (77).

There is no significant difference in the resistance level during and after the expansion. Researchers stated that the main resistance to expansion was caused by other bones connected to the maxilla, not by the midpalatal suture (54).

In a study of Rhesus monkeys it has been reported that the rapid maxillary expansion causes replacement in the lambdoid, parietal, midsagittal sutures and sphenooccipital synchondrosis. (68).

The resistance to sutural opening is not caused by the midpalatal suture but by the sphenoid and zygomatic bones. The pterygoid plates of the sphenoid bone, located posterior to the maxilla, do not have a suture to allow lateral displacement between them despite their bilateral positioning. The pyramidal processes of the palatine bones are connected to each other by pterygoid plates. This limiting effect of pterygoid plates of the sphenoid bone significantly reduces the sliding of the palatine bones in the sagittal plane (37).

In a study, the forces created by Haas, Hyrax, minne-expander, quad-helix and removable expansion devices on the 3-D photoelastic skull model are measured. It has

been reported that the most orthopedic force occurs with Haas, Hyrax and removable expansion appliances. The least force occurs with the minne device, but even this force has an orthopedic effect. It has been reported that quad-helix appliance forms orthodontic forces rather than orthopedic force. It was observed that the forces formed by Haas, Hyrax, minne-expander devices were concentrated in the front of the hard palate and the initial effects of the application were seen in the area between the central incisors and spread towards the incisive foramen. The stress is spread along the intermaxillary suture towards the junction of the palatine bones with activations. The forces are transferred from this region to the zygomatic bone and the zygomaticomaxillary and zygomaticotemporal sutures are affected. The forces are then spread to the medial wall of the orbit, the areas where the nasal and lacrimal bones meet, and the pterygoid plates of the sphenoid bone (14).

The biomechanical effect of rapid maxillary expansion to craniofacial structures was examined by finite element analysis (FEM). The maxilla with 10 mm expansion showed that the highest stress level was formed in the pterygoid plate of the sphenoid bone near the base of the head. In addition, zygomatic bone, the outer wall of the orbit, the outer wall of the nasal cavity and the molar and canine areas of the maxilla were also found to have high stresses. Based on the findings, it is argued that the resistance to expansion is caused by peripheral skeletal structures, especially pterygoid laminae of the sphenoid bone rather than the midpalatal suture (79).

When the maxilla starts to separate, the zygomatic processes show some resistance to expansion; however, it was stated that the suture system allowed the displacement of the expanded structures. Furthermore, it has been reported that pterygoid plates tend to bend when the pressure is applied and that the resistance to bending increases significantly in the areas near the cranial base, which are more rigid (79).

2.7. Bone Tissue

Bone is a type of connective tissue with high level of vascularity and innervation consisting of calcium and phosphate crystals within the collagen structure. Basic functions of bone tissue are; to provide form and rigidity, to support other tissues and vital organs, to protect these tissues and to provide the ability to move (80).

Bone tissue consists of bone matrix, fibers and bone cells formed by the calcification of intercellular fluid (81-83). 33% of the dry weight of bone is organic compounds and 60% is inorganic components. The organic components of the bone tissue are Type 1 collagen fibers in the fibrous structure, which constitutes 90% of the structure; amorphous sulphate and sulphate-free glycosaminoglycans (GAG) and glycoproteins, which make up 10%. (84, 85)

Sulphate-containing GAGs are called proteoglycans and their derivatives are chondroitin 4, 6 sulfate and keratan sulfate. GAG without sulphate is called the hyaluronic acid. The glycoprotein content of bone consists of osteonectin, osteocalcin, osteopontin and gamma carboxyglutamic acid (84,85).

The inorganic structure of the bone is formed by 95.5% calcium phosphate, 10% calcium carbonate and a small amount of calcium fluoride, hydroxide, sulfate compounds, magnesium and alkali salts. Calcium and phosphorous combine to form compression-resistant hydroxyapatite crystals. Structures that ensure that the bone does not break easily are the collagen fibers when hydroxyapatite crystals provide stiffness to the bone tissue (86-90)

The canal through the bone matrix contain living cells that comprise 15% of the dense bone weight. Some of these cells are the osteoclasts which serve to break down the old bone matrix acting like macrophages, while the osteoblast cells resemble fibroblasts and accumulate the new bone matrix, and through this cycle the continuous renewal of the matrix in the bone is ensured (91).

The mature bone consists of two types: cortical (compact) and trabecular (spongy) bone. The amount of cortical bone in the body is 75% while the amount of spongy bone is 25%.

The cortical bone, which is dense, very hard, resistant to external forces and has regular structure, consists of matrix lamellae around the channels called the Havers system. These lamellae consist of cavities called lacuna, and the lacunae contain bone cells called osteocytes (80).

The compact bones form the inner and outer surfaces of the flat bones and the outer surfaces of the long bones in the body while the spongy bone is weaker and softer

than the compact bone. This stress-resistant bone contains the bone marrow (82,83).

The soft, irregular and more elastic trabecular bone consists of interconnected trabeculae. Nutrition in cortical bones occurs by the Havers system containing lymphatic and blood vessels, nerve and connective tissue, while the trabecular bone is diffused through extracellular fluid (92).

Generally, each bone is composed of cortical bone surrounding the trabecular bone. The cortical bone is covered with periosteum. This membrane consists of two layers; the outer layer is fibrous while the internal layer has bone-forming potential.

The inner surface of the cortex is covered with a structure called endosteum. The bone resorption mechanism starts from this surface. Periosteum and endosteum are composed of osteoblasts and osteoclasts, and these cells are involved in bone formation and destruction while remodeling, growth and repair events continue in harmony (80).

2.7.1 Bone Structure Cells

Bone cells are located within the bone tissue itself or in the mesenchymal stem cell (MSC) rich conjunctival stroma of the bone marrow (87). Friedenstein (93) reported five types of different cells, originating from the stem cell, in response to different molecular signals initiating the activation chain. These are; fibroblast, osteoblast, chondroblast, adipocyte and myoblast.

Osteoprogenitor cells, osteoblasts, osteocytes and osteoclast cells are involved in the formation, destruction, and maintenance of bone structure.

2.7.1.1. Osteoprogenitor cells

Osteoprogenitor cells are the main production cells of bone tissue and are of mesenchymal origin. Osteoprogenitor cells are found in endosteum, inner layer of periosteum and Havers channels. These cells are activated and multiplied by the mitotic activity during the growth and injury periods of the bone and transformed into osteoblast cells (83,94,95).

2.7.1.2.Osteoblast cells

Osteoblasts are approximately 20-30 μm in size and are multi-layered cells. They are produced from mesenchymal stem cells in the bone marrow or endosteum, periosteum and perivascular pericytes (96). They have cytoplasmic extensions to communicate with adjacent osteoblasts. These extensions reach the osteoid network through the matrix (87).

Osteoblasts are the main cells responsible for the construction of bone matrix, and they show a dark basophilic cytoplasm with large nuclei in the electron microscope. They have well-developed Golgi and endoplasmic reticulum (83).

2.7.1.3.Osteocyt cells

The activities of the autocrine and paracrine hormone systems in the body are regulated by surface receptors that detect their commands. In addition, parathyroid and thyroid hormone (97) growth hormone (98), insulin (99), progesterone (100) and prolactin (101) hormones are specific for surface receptors and their activities are regulated by these hormones.

Osteoblast cells, which have a life span of 1 week to 10 weeks, are destroyed by apoptosis and converted into mature osteocyte cells. These cells function in the activation of remodeling (87,102).

Osteocytes are mature bone cells that develop from osteoblasts. These cells can survive for years by forming more than 90-95% of the skeletal structure (103).

Osteocytes in lacunae are smaller in size than osteoblasts. The transfer of substance in the bone tissue is provided by a large number of thin cytoplasmic extensions of osteocytes that extend in the canaliculi (104).

There are ideas that osteocytes are effective against mechanical stimuli and that they are responsible for maintaining bone mass (105,106,107). These cells are thought to stimulate osteoclast cells and improve remodeling by responding to stress in bone tissue (108).

2.7.1.4. Osteoclast cells

Osteoclasts are giant cells with approximately 20 to 50 nuclei responsible for bone resorption (87). These cells, which are 100 μm in diameter, make up less than 1% of all bone cells that form the skeletal system. Osteoclasts are in the mononuclear phagocytic system because they are formed by the convergence of monocytes from the blood and they are located in the cavities of the bone called “Howship lacuna”. The number of these cells increases in active bone production (109).

The membranes of the osteoclast cells have two important characteristics: the rough surface where the resorption occurs and the clear surface where the microfilament-rich integrins are located that provide binding to the matrix. The osteoclasts move towards the resorption area and adhere to the surface of the mineralized tissue by a rough surface and cover the edges of this area with integrins (87). On these connecting surfaces there are pit surfaces showing the presence of osteoclasts (109).

Osteoclasts secrete acid collagenase and proteolytic enzymes that affect bone matrix. Thus, while releasing the calcified basic substance, they play an active role in the phagocytosis of the residues that occur during resorption (90,110).

2.8. Osteogenesis

Ossification, also called bone formation mechanism, is performed in two ways; intramembranous (direct ossification) and endochondral (indirect, cartilage ossification) ossification. Direct ossification is caused by connective tissue and indirect ossification by the involvement of cartilage tissue. Regardless of which type of ossification, bone tissue made by osteoblasts is the primary immature bone tissue. This bone tissue is then replaced by mature lamellae bone tissue (82,83).

2.8.1. Intramembranous Ossification

The entire frontal and parietal bones in the skull, some parts of the occipital and temporal bones, the compact parts of the short and long bones, and some of the upper and lower jaw bones are ossified by intramembranous ossification. In this type of ossification, which occurs as a result of direct mineralization of the matrix secreted by osteoblast cells, mesenchymal cells migrate to the membranous ossification area and adhere to this area. With mesenchymal tissue vasculature and transformation of mesenchymal cells into osteoblast cells, these cells begin the production of collagen and proteoglycans to initiate bone matrix release. Calcium and phosphorus ions enter the osteoid tissue from capillary vessels and form calcium phosphate molecules via alkaline phosphatase secreted by osteoblast cells (104).

Erdoğan, Junqueira and Tekelioğlu define the area called the ossification center as where calcium ions are subsided into the amorphous substance and converted into osteocytes. The incorporation of many ossification centers within the same period of time creates bone tissue in the trabecular structure. Mesenchymal cells migrate into the connective tissue between ossification centers and differentiate into bone marrow cells. As mesenchymal cells continue to form osteoblasts by proliferating by continuous mitotic division, ossification centers increase. These centers fuse together to form the whole of the bone tissue (81,82,83).

2.8.2. Endochondral Ossification

Endochondral ossification is the transformation of short and long bones from hyaline cartilage to bone tissue. After the development of the hyaline cartilage of the developing bone structure, the chondrocyte cells in this draft expand and are degenerated. Endochondral ossification occurs with the synthesis of bone matrix as a result of the use of the calcified cartilage matrix as a support for the blood vessels and osteoprogenitor cells. Proteoglycans in cartilage tissue and cialic acid in capillary endothelium push each other due to the negative charge , to prevent vascular formation. Due to the mineralized matrix tissue and calcium ions released into the environment, interaction occurs and formation of vascularization occurs (111,112).

Ossification begins with the conversion of mesenchymal cells in the inner layer of the perichondrium layer in the diaphyseal region of the cartilage model to osteoprogenitor cells, and these cells into osteoblast cells overlying the cartilaginous septum with bone matrix. Overlying osteoblast cells form bone lamellae. Osteoblast cells trapped between these lamellae also become osteocytes. In this way, a cylindrical bone band is formed between the cartilage and the periosteum in the diaphyseal part of the cartilage model. Since the formation of the cylindrical structure prevents the feeding of the cartilage by diffusion, the structure of the chondrocyte cells begins to deteriorate while the thin cartilage matrixes remain in the field. With the activation of osteoclast cells, the holes called “foramen nutritium” are opened on the bone band, through which periosteum cells will enter the tissue. Thanks to the veins passing through these foraminae, the alkaline phosphatase, which passes through the chondrocytes to the cartilage matrix, joins the calcium and phosphorous ions into the cartilage matrix and an ossification center occurs in the diaphysis (81,83).

Erdoğan, Junqueira and Tekelioğlu describe that, as the endochondral ossification crosses the diaphyseal area and approaches the epiphysis, secondary ossification centers begin to appear in the epiphyseal areas. Among the old formed and newly formed bone fields, a cartilage disc called the epiphyseal plate remains. The chondrocyte cells of the epiphysis plates continue to form cartilage tissue by dividing and multiplying by the direction of diaphysis, while this cartilage tissue is replaced by bone tissue. Upon completion of bone elongation, the ossification of the epiphysis plates occurs and bone formation is complete (81,82,83).

2.8.3.Ossification in Dentofacial Sutures

Adjacent bones are connected to each other by the sutures which consist of connective tissue strips. The periosteum layer overlying the adjacent bones continues in the suture. The top layer of the periosteum, which is a type of connective tissue, is a fibrous layer and consists of fibroblast, collagen fibers and neurovascular bundle. The bone-facing surface of the periosteum is the cambium layer of the bone cells. The cambium layer is the layer where osteoblast cells are multiplied and converted into Sharpey fibers by precipitation of calcium salts onto collagen fibers secreted by these

cells, and where bone is made. In the sutures, adjacent bones have a cambium layer and a capsular layer consisting of fibrous tissues.

Both capsular layers comprise an intermediate layer with blood vessels therebetween. Thanks to the cambium layer, the two adjacent bones that form the suture continue to grow separately. These bones can grow at the same rate as one another, or one can grow more or less than the other (10).

2.8.4. Healing of Fractured Bone Structure

Bone healing takes place in three stages: inflammation, repair and remodeling (85). When damage to the bone occurs, the fibroblast cells migrating to this region and the fibrin network formed together form the blood clot by starting to organize the capillary structures. The formation of this structure in the form of a connective tissue is called the inflammation stage. This structure occurs within 7-10 days. In the repair phase, also called callus formation, mesenchymal osteoprogenitor cells in the periosteal and endosteal layer of the bone begin to form in the healing area with chemotactic signals (113).

The endochondral ossification of the small cartilage particles that occur in the fracture or damage area produces primary bone. At this stage the cartilage is replaced by the woven bone. This soft callus, which occurs between the seventh and twelfth days, is tasked with bringing together irregular bone fragments and leads to the development of secondary callus. With the formation of secondary callus between 20 and 60 days, woven bone turns into compact lamellae bone. Cells transformed into osteoblasts form bone trabeculae in areas where blood supply is sufficient. In this way, the outermost layer consists of bone-forming (osteogenic) cells, the middle layer of cartilage tissue, and the deepest layer of bone trabeculae (114,115).

As the healing progresses in the bone tissue, the callus begins to be replaced by mature bone cells and the trabecular structure begins to be organized to meet the functional needs. The forces acting on the bone during bone repair also help reshape the callus. In this period, called remodeling, osteoblasts produce new bone coverslips, while resorption by osteoclastic activity occurs. When the primary tissue of the callus is resorbed over time and replaced by the secondary bone, the original structure of the bone

is regenerated (116,117).

Studies have shown to have a positive effect on bone tissue repair by external application of growth hormone, type I collagen, calcium, C and D vitamins (111,112).

2.8.5. Bone Remodeling Metabolism

In a healthy tissue, the amount of bone production is greater than the rate of destruction, and it grows while maintaining the bone shape. In the remodeling phase, stress forces applied on the long axis of the bone create an electromagnetic field, and the bone cortex and bone marrow cells are continuously maintained. As a result of the applied mechanical stress, the inner side of the bone is loaded with positive and the outer side with negative electricity. Osteoclastic activity on the negative surface of the bone destruction occurs, while on the positive surface bone formation occurs with osteoblastic activity (116,117).

Biomechanical control mechanism of bone formation is provided by physiological response of skeletal adaptation as a result of functional loading. In this case, when the bone is atrophied by catabolic mechanism, apposition is formed by the hypertrophic response of the anabolic mechanism (118,119,120).

2.9. Structure of the Maxilla and the Midpalatal Suture

Maxilla; is a structure consisting of paired bones and is involved in the structure of the oral cavity, nasal cavity, orbita, fossa infratemporalis and fossa pterygopalatina.

Palatinal bone is also a paired bone and is involved in the structure of the hard palate and nasal cavity. It is bordered by the maxilla, sphenoidal bone, concha nasalis inferior, vomer, with the ethmoidal bone and on the opposite side by the other palatinal bone. The 3/4 front section of the hard palate (palatum durum) is formed by the maxilla on both sides, and 1/4 posterior part is formed by the palatine bones (43).

Melsen (121) examined the structure of suture from the birth to the late adulthood in a cadaveric study and from the vertical sections he discovered that the suture was Y-shaped and bordered with vomer by the palatal processes in childhood. In adolescence, the junction of the bones was more superior and T-shaped, while the interpalatal part showed a snake-like image. In the adult period, an interdijitation occurs in the suture area and the bones are mechanically locked. Bone islets are formed around the bone (121,122).

Having knowledge about the structure of the midpalatal suture and the ossification metabolism, the researchers worked on shortening the time period of ossification and also lowering the risk of relapse. Many different biological substances or treatment options have been researched on ossification of the expanded midpalatal suture such as ED-71 (active vitamin D analog), thymoquinone, low-power laser irradiation, recombinant human bone morphogenetic protein-2, dietary boron, zoledronic acid, propolis and hyaluronic acid (123-129).

2.10.Hyaluronic acid

HA was first obtained by the researchers named Meyer and John Palmer in 1934 from the vitreous body in the cow's eye. Due to its bright and transparent appearance, it is named after uronic acid which means "glassy" in Greek (130). In the present age, we can be derived from additional sources such as rooster combs, shark skin, and many microorganisms (131).

The idea of usage HA in medicine can be dated back to 1940s , Balazs and Piller focused on the experiments about bone and cartilage tissue healing (132). Also in 1960 the usage of HA as a viscous material for vitreous of the eye was evaluated by Balazs (133). This researches became the framework of 'matrix engineering' and open way to regenerative and development processes of both the circulatory and musculoskeletal systems (134).

In 1986, Balazs (135) proposed the name hyaluronan as an alternative to HA. Today, the current hyaluronan preparation is sodium salt. Meyer isolated HA from the chicken comb, as Kendall et al. isolated it from bacteria (136).

HA, which is frequently used in the fields of orthopedics, dermatology and ophthalmology, is among the most commonly used materials in recent years due to its anti-inflammatory, antibacterial and moisturizing effects in the field of dentistry (137-139). The first commercial usage of HA was in 1980 as an ophthalmic viscosurgical device solution, trademark was Healon[®] which is base model of our experiment material Healon GV[®] (139).

HA plays an important role in various biological cycles, including wound healing, chondrogenesis, immune response and cell migration (140,141). In addition, HA affects the early biochemical and physiological stages of osteogenesis as continuous extracellular matrix component in bone morphogenesis (142).

2.10.1. Chemical Structure of Hyaluronic Acid and The Level of HA in Body

HA is a non-sulfate, flat-build unbranched GAG with high molecular weight (HMW) and low molecular weight (LMW) derivatives, also called hyaluronate or hyaluronan. The molecular weight of hyaluronan is usually in the order of 10^6 to 10^8 (192,195). This polysaccharide is a macromolecule (143-146) linked by β -1,4-D-gluconic acid and the β -1,3-N-acetyl-D – glucosamine chain ring. Other GAG types such as chondroitin 4.6 sulfate, keratan sulfate are synthesized in the golgi body, whereas HA is synthesized in the cytoplasm of the plasma membrane. It also differs from other GAGs due to its ability to contain sulphates and to covalently bind with proteins (144). 20-30% of HA's destruction metabolism occurs in the skin and joints, while the other is done by lymphatic route. HA's destruction in the dense tissues such as bone and cartilage cannot be achieved by lymphatic drainage, but it is performed together with collagen and other proteoglycans. While the half-life of HA varies between 2-5 minute and 2-3 days, 85-90% of the part of the bloodstream is disrupted in hepatic cells in the liver, 10% in the kidneys and the rest is excreted in the urine (131,147).

Within a healthy 70 kg human there is approximately 15 gr of HA (148). HA found in the extracellular matrix (ECM) of all tissues has no known side effects (146,149,150). Approximately 50% of HA is found in the skin, lip and mucosa, 25% in the skeletal system and joints and the rest in the muscles and internal organs, while the highest concentration is found in connective tissues such as umbilical cord, synovial fluid,

skin and vitreous body. The lowest concentration was reported in the blood serum (147,151,152).

The total turnover of hyaluronan in serum is in the order of 10–100 mg/24 h. The normal concentration of hyaluronan in serum is >100 µg/l with a mean of 30–40 µg/l. High serum levels have been noted in liver cirrhosis (impaired uptake in the liver) and rheumatoid arthritis (increased synthesis in the tissues). (5)

Human synovial HA averages about 7 million Dalton per molecule or about twenty thousand disaccharide monomers, while other sources mention 3–4 million Dalton. As one of the chief components of the extracellular matrix, hyaluronan contributes significantly to cell proliferation and migration, and may also be involved in the progression of some malignant tumors (153).

The fact that most of the amount of HA in the tissues and blood in the resting period is cross-linked with collagen and other HMWs indicates that the amount of free density is low, whereas in tissue injury and inflammatory conditions the density of HA in tissues increases considerably (154,155). It has been reported that all HA in the body destroyed and re-synthesized every day for one third (156). The level of HA in human blood serum varies between 10-100 µl / L (150).

While it is 0.05-3 mg/ml in adult tissues, it may be as high as 12-20 mg / ml in some embrionic tissues (157). HA is found a certain amount in lung, kidney, brain and muscle, while a small amount in the liver (147).

2.10.2. High and Low Molecular Weight Hyaluronic Acid

HA with different MW is reported to between 1×10^5 to 10^8 Da (Dalton, MW unit) (139,145,158-160). There are studies (136, 161-169) and systematic reviews (170,171) comparing the effects of HMWHA and LMWHA on bone, cartilage and different soft tissues. Various molecular weight of HA shows different biological effect. HA's with a molecular weight of >1000 kDa have anti-apoptotic activity whereas HA molecules with a size of <200 kDa have a strong pro-inflammatory stimulus (172). There are studies (158,173-181) in which the effects of HMW on bone and cartilage tissue are evaluated

alone, and studies evaluating LMW HA alone are performed (138,182,183).

As the MW increases the viscosity and viscoelasticity rise up progressively. Physico-chemical properties of HMWHA allow to modulate tissue hydration and osmotic balance creating a hydrated and stable ECM (139). In the study of Garcia-Abuin, it was noted that viscosimetric behavior, the intrinsic viscosity of HA is 2.12 dL/g, which is similar to human whole saliva (184). It has been shown that high temperatures and elevated concentrations of electrolytes decrease the viscosity of HA. Decrease of viscosity can diminish the lubricant and shock absorbing properties of HA (185).

Different MW forms of HA preparations that have been used in osteoarthritis (OA) therapies for many years and their efficacy have been investigated by meta-analysis studies. In 2003, Lo et al. (170) and in 2005 Arrich et al. (171) evaluated systematic reviews. Lo et al. (170) examined the studies with 7 different MA preparations, reported that HMWHA preparations were more effective than LMWHA preparations, but the results were difficult to interpret due to the heterogeneous distribution between studies. Arrich et al. (171) found no difference in the effects of MA and HA preparations. Due to the HMW structure of HA, it is indicated that it can bind to cell surface proteins and other matrix proteins by acting as a filter for macromolecules (158).

In vitro studies have demonstrated that HMWHA increases the isolated proteoglycan aggregation by forming a plug, synthesis of sulfated GAG, stimulates fibroblasts and new HA formation in the culture medium; inhibits active macrophage and leukocyte chemotaxis, IgG (Immun Globulin G) and phagocytosis by aggregated neutrophils and enzyme release and acts as a free radical scavenger (173,178-180).

Iwata et al. reported that the concentration, MW and viscosity of HA decreases in diseased tissues (136). They also reported that the biological function was at the highest level when the molecular structure of HMWHA was intensely polymerized and that the HA oligomers with LMW were not important except the vascularization effect in tissues.

Asari et al. (167) in their study on dogs, showed that LMWHA (840.000 Da) showed higher permeability to synovial membrane compared to HMWHA (2.7 MDa). It is stated that the MW is not the single factor for the increase of the synthesis of HA in the tissue and there is no study on human about this topic.

Huang et al. (186) observed that HMWHA exhibits antibacterial, antiinflammatory and even antioxidant properties since it is degraded by reactive oxygen species and free radicals. On the other hand LMWHA shows immunostimulatory and proinflammatory behaviors (187).

2.10.3. Local and Systemic Effects of HA in Metabolism

HA in all parts of the body has been reported to undertake different tasks as a result of the studies. HA is involved in T cell signaling, wound healing, morphogenesis and matrix organization; it is also reported that HA plays a role in the regulation of TGF-B1 (carrier growth factor) by providing fibroblast and myofibroblastic proliferation (159,188-193).

HA; which was considered as a structural and passive component of ECM in the first years of research, has the ability to reduce water molecules in the environment by diffusion or condensation on the cell wall (hygroscopic structure) and because of this ability HA is effective in all healing, cell renewal, cell signaling and adhesion to tissues processes. (151,174). HA is capable of holding 1000 times more water than polymers (144).

HA can be found in active cell growth regions and plays a role in cell growth by binding to multiplying cell receptors (154). It is also known to accelerate the process of re-epithelization in wound healing by increasing collagen fibril formation (146,194,195).

Cell surface receptors such as CD44 and RHAMM (CD168) play a role in cellular intercommunication. HA provides intercellular signaling by producing cell surface aggregates with the contribution of proteins such as hyaladherin (145,182).

The interaction of HA with other proteoglycans gives stability, flexibility and surface filling properties to ECMs of all tissues (154,174).

This communication between macromolecules determines the mobility of the substances in the interstitial area and moisture density in the tissue (154).

HA is found to have a higher rate in the fetus than in adults and it contributes to tissue healing without scarring in fetal tissue injuries. This was determined based on the fact that HA levels in adult tissue return to normal within 7 days while in fetal tissues they stay at high molecular levels up to 3 weeks (144).

Since HA is present in the cartilage in fetal tissues, it is known to help the nasoalveolar molding process with its moisturizing effect in babies with cleft palate in the early period (196,197).

HA is often used in the treatment of radiodermatitis, decubitis and also nasal mucosa ulcers because of its granulation tissue and re-epithelialization process enhancer effects (198).

HA-containing products are often used in cosmetic and dermatological fields. HA is the primary substance of connective tissue intercellular matrix and because of its higroscopic features, it has an important task in providing the turgor pressure of the skin. This substance, which is found excessively in fetal skin, decreases in the body with age (149).

HA has a key role in wound healing processes in all mineralized and non-mineralized tissues (199). Wound healing phases respectively are inflammation, granulation, re-epithelialization and remodeling. HA has many functional roles in all these cellular and ECM activities (200). Following injury, it is reported that the amount of HA in the wounded area increased and then the concentration of HA decreased over the next few days (200).

With the onset of inflammation, the amount of HA increases significantly in this area. This increase occurs in order to initiate the repair process of tissue inflammation, migration of ECM cells to this area, and to be established a temporary structural skeleton by HA by forming the fibrin clot relationship. Therefore, the first stage of the formation of inflammation in the healing process is regulated by HA (155,199,201).

In the second stage of inflammation, the formation of granulation tissue and matrix takes place. At this stage, inflammation is quite high due to the destruction caused by matrix degradation enzymes and reactive oxygen metabolites (202). The matrix of

granulation tissue is also rich in HA. At this stage, HA provides cellular organization of granulation tissue, as well as facilitates migration of cells to this area (155).

During the formation of granulation tissue, which starts with the migration of cells to the field, HA communicates with cells and forms a moist matrix structure due to its physical and chemical properties. Here, by establishing a connection with certain cell receptors, it regulates the movement mechanisms of the cells and synthesizes in the plasma membrane and facilitates the migration of the cells thanks to the dynamic force created in the ECM environment in which it is released (199,202).

The increased level of HA in the cell proliferation stage following the formation of granulation tissue allows the fibroblasts to separate from the matrix and initiate cell division. Studies have shown that an increasing amount of HA facilitates the separation of cells, but it has not a direct effect on cell division. This macromolecule, which acts indirectly during the cell proliferation stage, plays an important role due to its effect on the separation of cells (199,202).

In order to achieve tissue repair in wound healing, the first inflammatory response should be alleviated in later stages. HA is effective in alleviating inflammation by preventing free radicals in the tissue matrix from damaging the cells and providing the necessary stable structure in the granulation tissue matrix (199,202).

2.10.4. Hyaluronic Acid in Human and Animal Studies

HA is used for treatment and researches on many different parts of the human and animals. The first of these are the eye, cartilage, bone tissue and skin. In ophthalmology HA has been studied generally in treatment of dry eye disease. Between %5-34 of people are affected by dry eye having symptoms; redness, burning, foreign body sensation, stinging, pruritus and photophobia (203). The most known treatment is the use of artificial tears made up of polyvinyl alcohol, povidone, hydroxypropyl guar, cellulose derivatives and HA. HA supply viscoelastic properties that can lubricate the ocular surface and reduce friction during blinking and ocular movements (204). In Gro, Childs & Piaton's experimental study, they found HA is naturally in physiological tear fluid and possesses

excellent moisturizing and mucus layer adhesive properties (205). Parallel to this study Fezza observed, the water retention and lubricant properties of HA beneficial for dry eye treatment (206).

In rheumatology, general use of HA is for osteoarthritis (OA) patients that cause limited mobility and stiffness. Risk of OA increase with age and obesity (207). Ishijima et al. worked on OA patients. Multi-center, open label, randomized study is operated and plan to compare the efficacy of intra-articular HA versus an orally used NSAIDs, Ixopropfen (208). Other open label, randomized, control trial studied by Hermans et al., They searched the effectiveness of intra-articular injection HMWHA on regular non surgical OA patients (209). Both researchers observed parallel results, HA groups shows significant improvements in pain, function and patient's global assessment (PGA).

Similar to present study there are a few researches on bone and cartilage tissue. Arpağ ve ark. (210), created bicortical cranial defects on calvarial bones of ten adult New Zealand rabbits and used three different types of graft materials (xenograft, xenograft+HA and autograft) to compare bone healing capacities. They preferred high molecular HA for the study. New bone amounts were statistically significant between all the groups. In the autograft group, residual graft volume was lower than xenograft and xenograft+HA groups. And it was lower in xenograft+HA group than only xenograft group. Their results show that high molecular HA improves the amount of healing in bone defects. But HA does not have any positive effect on the quality of the newly formed bone.

Engstrom et al. (174) applied locally HA with syringe in the form called “Rooster compound”, which is a derivate of HA with HMW (high molecular weight) on the teeth with periodontal damage after surgical and non-surgical interventions. They observed more new bone formation in the HA group than in the non-HA group. In their clinical findings after 12 months of follow-up, they reported a significant decrease in periodontal pocket depths in the HA group compared to the non-HA group, and the difference in bone level between the groups was less than 1 mm in radiographic findings.

Sasaki and Watanabe (176) formed cavities in the femoral area of rats, injected regional HMWHA (NRD® 101, Japan Roussel Co. Ltd., Tokyo) (1900 kDa MW) and

examined ossification in 14 days. They made the investigation with light microscope and scanning-electron microscope. According to this, new bone formation started to be seen on the cavity floor and walls starting from the 4th day in the HA group. They reported that the cavity was completely filled with trabecular bone and that the granulation tissue formed in the first period was rapidly replaced by new bone marrow tissue. In the control group, the newly formed bone tissue replaced with completely trabecular bone at the end of day 14.

Stanciková et al. (162) gave HA orally in different doses and different MWs (1.62 MDa 1mg / kg / day and 0.5mg / kg / day, 0.75 MDa 1 mg / kg / day) overectomy applied rats for 8 days. They reported that it prevents bone destruction after overectomy in HMWHA groups (1,62 MDa MW), and maintains bone density by increasing bone mineral content.

Zanchetta et al. (183) applied HMWHA (HE 800) produced from bacterial exopolysaccharides to the bone cavities formed on the parietal bones of rats, leaving the bones of the control group empty, and reported that the cavities were completely filled with bone after 21 days.

In another study by Zanchetta et al. (138), they filled the parietal bone cavity of rats with HE 800 (800 kDa), which is a derivate of HA produced from bacterial exopolysaccharide. They filled the other parietal bone cavity of the same rat with collagen as control group. At the end of 15 days, radiographic and histological sections were evaluated. They reported that bone density was $95.9 \pm 6.2\%$ in the study group and $17.8 \pm 18.1\%$ in the control group.

Aslan et al. (177) applied HMWHA (4 MDa MA in 4 ml gel form) together with bone graft locally to the bone cavity formed in the rabbit tibia (Healon® Pharmacia & Upjohn, Sweden). They stained the 5 µm thick sections with hematoxylin eosion stain (HxE) and examined histopathologically under light microscope. When the results were compared with the control group with HA alone, they reported that bone formation was significantly higher in the HMW HA+ bone graft group.

Pirnazaar et al. (143) used HA derivatives with low (141 kDa), moderate (757 kDa), and high (1300 kDa) MW to investigate the bacteriostatic effect of HA. They found the bacteriostatic effect significantly higher in 6 bacterial groups where HMWHA was applied than other groups. MMWHA was found to be effective in 2 groups, while it was found to be partially effective in 4 groups. Based on these results, they reported that HMWHA can prevent the spread of bacteria during surgical procedures.

Kim et al. (211) investigated if HA improves the bone formation in extraction sockets. The experiment was carried out on six beagle dogs. They hemisected the third molars and extracted the distal roots bilaterally. They induced periodontal and endodontic lesions on the mesial roots of the hemisected teeth. 4 months later, the mesial roots were also extracted. They applied HA into the sockets of the mesial roots of the test group. Formation of mineralized bone amount was significantly more in the test group. The researchers reported that HA may be helpful in infected bone healing because of its bacteriostatic and anti-inflammatory effects.

Zou et al. (212) used pig bone marrow in their study to investigate the effect of hyaluronan on osteogenic differentiation. They researched whether prolonged presence of high concentration (4.0 mg/mL) 800 KDa HA and pretreatment with HA can modify osteogenic differentiation of pig bone marrow stromal cells (pBMSC). They measured cell proliferation and mineralization. Expression of differentiation-related genes was evaluated by means of real time reverse transcription polymerase chain reaction (RT-PCR). They observed that HA increased cell proliferation on day 7. Study results suggest that exogenous HA stimulates endogenous HA, which together may play a synergetic role in osteogenic differentiation under osteoinducing conditions although gene expression was inhibited at the early stage.

HA also used in dermatology and cosmetics that applied as dermal fillers, anti-wrinkle agents and in tissue regeneration. HA based fillers seem to be ideal because of their low immunogenic reaction and relatively long lasting effect (213).

Kandhari and Rahman (214) studied about HA's filler capabilities in the correction of congenital and acquired lip asymmetries. They examined the effectiveness of HA filler injections. The patients were initially injected between 0.4-1.6 ml of HA.

After the first application, they injected 0.4-0.8 ml HA 3 months later and they repeated injections every six months. There were no any bruising, edema or complication noted as expected. Significant results were taken about patient's lip fullness as well as effectiveness in filling their scar.

Another multicenter randomized double blind study was performed to nasolabial folds (NLFs). The study compared dermal filler VYC and a non animal HA filler. 123 patients were treated and were followed up after six months. Outcomes were assessed on the NLF Severity Scale, post and pre photographs, as well as patient satisfaction. Improvement of esthetic were seen in both groups (215).

In dentistry limited studies were performed about dental papilla reconstruction and rapid palatal expansion cases. Abdelraouf et al (216) performed their experiment to eight patient with deficient papilla. The patients were randomly divided into groups that all received a 0.1 ml HA gel injection or 0.1 ml saline injection. Between 3 to 6 weeks intervals patients received a total three injections at baseline. Visual analogue scales and pre-post injection photographs used for assessing patient satisfaction. Three months after injection, the HA group had a reduction of black triangle surface of %36.5. Moreover, six months later reduction was increased to %45. At the same time there was no statistical changes seen at saline group.

Parallel to present study, Bekçi (217) studied on expanded midpalatal suture area and used different molecular weight HA on rats. Twenty four rats divided into three groups and after expansion each group received 50 µl HMWHA, LMW HA and saline solution. Ten days after injection histomorphometric evaluation was performed. HMWHA showed a higher osteoblast and capillary activation but there were no any significant differences between LMWHA and control group.

2.11. Use of Rats as Experimental Animals

Rats are the most widely used organism in medical research, and the successful isolation of rat ES (embryo-derived stem) cells will quickly expand their utility. As a model of human disease, the rat offers many advantages over other organisms. The rat is

an excellent model for many diseases and there are a variety of genetic stocks that are ideal for most studies. The physiology of the rat is easier to monitor. Moreover, in many cases, the physiology is more like the corresponding human condition. The size of the animal enhances its use as a disease model, not just because of the ability to perform surgical procedures, but also because of the proportional size of important substructures in organs that affects both how much of the organ is involved in an experimental lesion and the distance effects of drug administration to specific anatomical areas. This is particularly important in the central nervous system. The rat is the primary model for mechanistic studies of human reproduction. For drug studies, the size of the rat enables serial blood draws. This is by no means a comprehensive list, but gives a sense of the depth and range of use for this animal model (218).

The metabolism of rats, dogs, monkeys and pigs have similarities to humans but they also have significant differences. The pigs are less preferred because of their difficulty of care and cooperation. The inclusion of dogs in studies has been restricted in recent years. Monkeys are also less preferred because of their cost and difficulty in maintenance. Rats are the most preferred animals in studies because they are easy to reproduce to host and to feed. They have low cost and their histological preparations are easier to do (219).

2.11.1.Dentofacial Structure and Anatomy of Rats

The rats, which are frequently preferred in the studies are adults with 2,5 to 3 years life span. They have an average weight of 300-350 g (220).

Andreollo (221) et al. stated that a day of rats life coincides with 30 days of human life. Other researchers reported that a male rat weighing 180-200 g corresponds to a male human individual of ages between 14-16, who have not completed the development of growth (125).

Unlike human beings, rats teeth are renewed for life and show elongation movement (222). There are 4 incisor teeth in the upper and lower jaws of the rats and 12 molars total, three in the right and left upper and lower jaws (223,224).

2.11.2. Bone Healing Metabolism of Rats

The rats' alveolar bones are denser than humans and they have less bone marrow cavity. Following the application of an orthodontic force, the resulting reaction in humans is after around 30-40 hours, while in rats within 6 hours and the results show similarity (225). In addition, due to differences in bone structure, cementoblast cells react faster than osteoblast cells for remodelling (226).

Misawa et al. (227) compared the effect of the age of the rats on the speed of alveolar bone construction. They stated that under physiological conditions, the bone formation rate decreased as the age of the rat increased. There was a statistically significant difference between 6 week and 30-40 week old rats.

Burstone and Schafer (228) reported that the midpalatal suture areas of young rats were approximately 20 to 60 μm thick. Li et al. (229) reported that fracture healing was completed in the 5 week period and the fastest bone formation was in 3 weeks.

3. MATERIALS AND METHOD

3.1 Animals and Groups

In this study, permission was obtained from Yeditepe University's Ethics Committee of Experimental Animals (Date: 02/06/2016 Approval number: 471). The experiments were carried out in Yeditepe University Department of Animals Research and Development Center (YUDETAM). Histomorphometric, histopathological and biochemical evaluation was held in Yeditepe University Department of Histology.

48 male 10 to 12 week old Sprague-Dawley rats were used in this study. The average weight was $361,25 \pm 42,03$ gr at the start. The weight changes of rats were measured 3 times during study.

Table 1. Shows mean and standart deviation of weight of rats in day 0, 5, 10 and 20.

Time	Begining of Experiment Day 0	After expansion Day 5	End of Experiment Day 10	End of Experiment Day 20
Mean and Standart Deviation	$361,25 \pm 42,03$ gr	$330,77 \pm 40,76$ gr	$334,34 \pm 37,33$ gr	$360,86 \pm 47,63$ gr

They were housed in 60 x 40 x 40 cm polycarbonate cages without bedding materials in a 12 hours light/dark room at the temperature of $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and fed with powdered pellets (expanded pellets, Stepfield, Witham, Essex, UK) with tap water ad libitum. Before the experiments, all animals controlled and general health confirmation taken by veterinary.



Fig. 3.1. Polycarbonate cages, identification numbers on cages

The 48 rats were randomly divided into 6 groups, each with 8 rats (control and experimental groups). Every 2 or 3 animal kept in the same cage and we marked their ears and tails for identification.

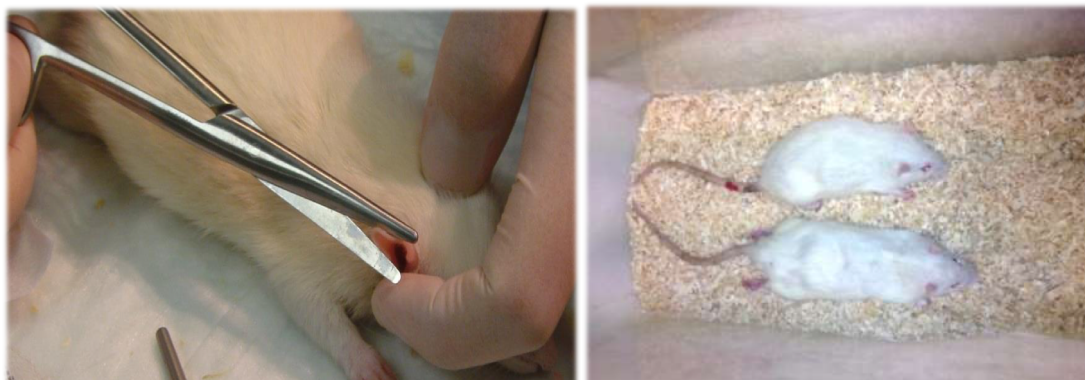


Fig. 3.2. Marking their ears and tails for identification.

The power analysis was performed with G Power ver.3.0.10 software (Franz Faul, Universitat Kiel, Germany) based on 1:1 ratio between the groups, a sample size of 48 animals could give more than %90 power to detect significant difference with a 0.40 effect size and at a level of significance of 0.05.

Table 2. Number of animals for each experimental group

	Low Molecular Weight HA (LMW HA)	High Molecular Weight HA (HMW HA)	Control Group (Isotonic Solution) (CG)
Short Term (10 Days)	8 Rats	8 Rats	8 Rats
Long Term (20 Days)	8 Rats	8 Rats	8 Rats

3.1.1. End Point Criterias of Experimental Animals for This Study

- Weight loss more than %15 of total body mass
- Behavioral abnormalities
- Reduced respond to environment
- Decision of veterinary because of humane reasons
- Broken tooth or appliance that we used in experiment
- Reject to water of food intake of animals

3.2. Materials Used In The Experiment

- 0,85 ml Sterile injector (Hayat A.Ş., Turkey)
- Ketazol ® (Richter Pharma AG, Wels, Austria)
- Rompun® (Bayer Helayh Care LLC., Cansas, USA)
- 0,014 inch stainless steel wires (American Orthodontics, USA)
- 0.8 mm width stainless steel ligature wire (American Orthodontics, USA)
- Orthodontic Pliers (Dentaurum, Ispringen, Germany)
- Low-speed handpiece
- Diamond burs (Comet, Reference Number 8392314016, Lemgo, Germany)
- Analog Force Gauge (Haag-Streit Diagnostics, Könitz, Switzerland)
- Acid etching (37% phosphoric acid, Ivoclar Vivadent, Liechtenstein)
- Bonding agent (Transbond XT primer, 3M Unitek, Monrovia, CA, USA)
- Transbond XT Light Cure Adhesive (3M Unitek Monrovia, CA, USA)
- Flowable composite (3M Unitek Monrovia, CA, USA)
- Digital caliper with a sensitivity of 0.01 mm (Max-Extra®)
- Low molecular weight hyaluronic acid AMO Vitrax II® (Lifecore Biomedical, MN, USA)
- High molecular weight hyaluronic acid Healon GV® (Pharmacia & Upjohn AB, Sweeden)
- Plastic containers for tissue samples (Fırat Plastik, Turkey)
- %0,9 Isotonic sodium chloride solution (Eczacıbaşı, Baxter A.Ş., Turkey)

3.2.1. Materials Used in Histological and Biochemical Investigation

- %10 neutral formaldehyde (Merck & Co., ABD)
- %85 formic acid (Tekkim Laboratory Chemicals, Tuzla, İstanbul)
- Tri-sodium citrate (Tekkim Laboratory Chemicals, Tuzla, İstanbul)
- Tricrom-Masson (Merck & Co., ABD)
- Paraffin (PatoLab, Esenler , İstanbul)
- Pato-Cassette Tissue tracking cassette (PatoLab, Esenler , İstanbul)
- Polylysine Slides (Sigma Aldrich, Missouri, USA)
- Cover Glasses 24x60 (Sigma Aldrich, Missouri, USA)
- Light green S.F. Yellowfish (Sigma Aldrich, Missouri, USA)
- Absolute alköl (Merck & Co., USA)
- Fuksin asit (Merck & Co., USA)
- Xylene (Sigma Aldrich, Missouri, USA)
- Asetik asit (Merck & Co., USA)
- İncubator (Nüve EN 055, Ankara, Turkey)
- Microtome (Thermo Scientific, Microm HM 340E, Massachusetts, USA)
- Microtomeblades (Feather®, R35, Japan)
- Paraffin Embedder (Leica, EG1160, Wetzlar, Germany)
- Light Microscope (Leica, DM750, Wetzlar, Germany)
- Blood Serum Hyaluronic Acid Level Indicator Set (Shanghai Yehua Biological Technology, Shanghai, China)
- Centrifuge Machine (Leica, Wetzlar, Germany)

3.3. Method

After weight measurements, expansion appliances were placed at the maxillary incisors of all the animals when they were under anesthesia with %10 Ketasol® 60 mg/kg/im, including 100 mg/ml ketamine hydrochloride (Richter Pharma AG, Wels, Austria) and %2 Rompun® 5 mg/kg/im, including 23,32 mg/ml xylazine (Bayer HealthCare LLC., Kansas, USA) combination applied intramuscular injection.

The expansion appliance consisted of helical springs which were 0,014 inch stainless steel wires. Springs were placed at the level of gingival papilla. Spring arms placed near to distobuccal corner of the upper incisor teeth, a hole was drilled in the both incisors with diamond burs and springs placed palatally.



Fig. 3.3. Drilling Hole and Preperation for Helix Spring



Fig. 3.4. Application of Helix Spring

Appliance activated on just one arm. A force gauge was used to investigate 0,5 N expansion force. Springs were not reactivated during the first 5 days of expansion period. On the 5th day of expansion, springs were removed and rectangular retention wire placed and fixed with composite materials during 10 and 20 day retention period. Due to continuous eruption of the rat incisors, retentive grooves on the upper incisors were prepared as gingivally as possible.



Fig. 3.5. (a) Measurement of Expansion amount after 5 days Expansion
(b) Retention with rectangular stainless steel wire

After expansion, tooth separation was achieved at least 2mm, measured with digital caliper at the beginning and end of expansion period. Separation was maintained

until the end of the experiment. Animals were randomly separated into six different groups of eight rats each; first two groups received saline solution, second two groups received HMWHA (Healon GV®, Pharmacia & Upjohn AB, Sweden), third two groups received LMWHA (AMO Vitrax II®, Lifecore Biomedical, MN, USA). All solutions were locally injected directly into midpalatal suture zone with 0,85 ml injector and 0,05 ml (50ul) solution was applied per animal.



Fig. 3.6. High Molecular Weight Hyaluronic Acid (Healon GV®)

Fig. 3.7. Low Molecular Weight Hyaluronic Acid (AMO Vitrax II®)

Fig. 3.8. Salin Solution

The animals in the subgroups of both control and experimental groups were sacrificed by overdose anaesthesia after 10 and 20 days from the retention period of expansion according to the groups they belonged. Overdose anesthesia includes ketamin and xylazine combination. After sacrifice, their maxilla were dissected and placed in plastic containers that includes %10 formalin at 4C° and their blood was directly taken and sent to centrifuge machine to obtain the blood serum.

3.3.1. Histological and Histomorphometrical Evaluation

After soft tissue dissection, maxilla were separated from the skull by means of surgical scissors. Tissue samples were fixed in 10% neutral formaldehyde in 0.1 mol of phosphate buffered saline solution (pH 5.74). Samples were decalcified with Morse solution (10% sodium citrate and 22.5 % formic acid) for 4 to 5 weeks. After sufficient decalcification was obtained, out of the area between second molars and incisors were cut out by surgical blade, washed under tap water one night and alcohol series applied for

dehydration and were embedded in paraffin. Paraffin blocks were sectioned longitudinally (10 μ m thickness) parallel to the long axis of each tooth, from mesial to distal aspects. Choosing the first section was done in a systematic random manner. Sampled sections were stained with Masson's trichrome technique.

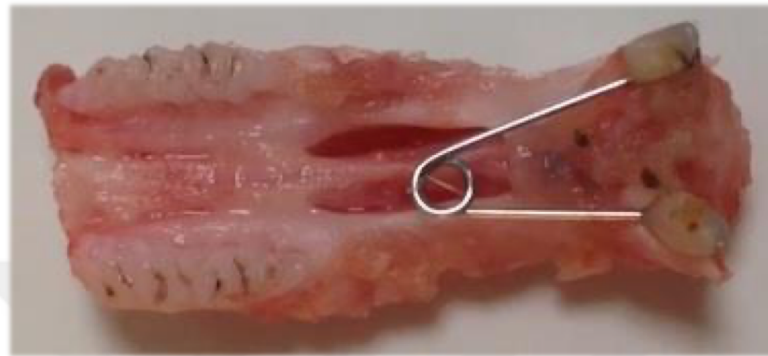


Fig. 3.9. Dissected rat maxilla



Fig. 3.10. Paraffin blocks were sectioned longitudinally (10 μ m thickness) parallel to the long axis of each tooth, from mesial to distal aspects.

Table 3. Tissue Fixation Stages

Stages	Time
Wash under tap water	1 night
%70 ethyl alcohol	1 hour
%80 ethyl alcohol	1 hour
%90 ethyl alcohol	1 hour
%96 ethyl alcohol	1 hour
%96 ethyl alcohol	1,5 hour
%100 ethyl alcohol	1 hour
Xylene 1	1 hour
Xylen 2	1 hour
Paraffin 1	1 hour
Paraffin 2	3 hours

Table 4. Stages of Masson's trichrome technique

Station	Time	Materials
Incubator	10 min	
Oven	3 min	
	3 min	Xylene
	3 min	Xylene
	5 min	Xylene
	2 min	%100 ethyl alcohol
	2 min	%96 ethyl alcohol
	2 min	%70 ethyl alcohol
	1 min	Distilled water
	4 min	Hematoxylin
	1 min	%1 acid alcohol
Wash	1 min	
	5 min	Acid Fuchsin
	1 min	Distilled Water
	5 min	Phosphomolybdic acid
	6 min	Light Green (Methyl Blue)
	1 min	Distilled water
	2 min	%1 acid alcohol
	1 min	%70 ethyl alcohol
	1 min	%80 ethyl alcohol
	1 min	%96 ethyl alcohol
Exit	1 min	%100 ethyl alcohol
Manuel	5 min	Xylene 1
Manuel	5 dk	Xylene 2

Histologically stained specimens were investigated in stereological work station, which consisted of computer controlled motorized microscope stage (Bioprecision, Howtrone, NY, USA), CCD Digital camera (Optronics Microfire 1600x1200P, Goleta, CA, USA), mikrokator (Heidenhein, Traunreut, Germany), display card (ATI FireGL Advance Micro Device, Camberly, England) and light microscope (Leica DM 4000B, Wetzlar, Germany). Volumetric measurements were made using Leica C Plan objective (magnification of x20). In order to analyse alveolar bone volume, Cavalieri estimator of Stereoinvestigator 11.0 (Microbrightfield, Williston, VT, ABD) software was used.



Fig. 3.11. Stereological work-station

Alveolar bone volume was determined using Cavalieri principle of previously mentioned stereological work station. The bone tissue in this boundary was defined as alveolar bone volume, whereas total soft and hard tissue in this area was defined as total volume. In order to estimate volume of each section, the maximum efficiency was obtained by using a point counting grid ($d=50\ \mu\text{m}$). The representative areas per point (a/p) were $2500\ \mu\text{m}^2$ for each point. After applying the point counting grid to the sampled sections in a systematic random fashion, the numbers of points hitting alveolar bone in the sample were counted. Results were used for the estimation of alveolar bone volumes using the following formula:

$$\text{volume alveolar bone} = t (\mu\text{m}) \times a/p (\mu\text{m}^2) \times \Sigma p$$

where, t is the mean section thickness; a/p represents the area of each point on the point counting grid; and Σp is the total number of points hitting the sectioned area.

In order to measure bone volume stereologically, a pilot study has been carried out regarding the region we will examine. The aim of this pilot study is to evaluate the desired histomorphometry and to have information about the tissue of interest. In addition, it provides rough estimations about the statistical significance of the study with the help of numerical data obtained after the measurements. Histomorphometrically, the measurement coefficients are to ensure that the Coefficient Error is less than 0.05 (2).

The results were evaluated on bone volume (mm^3), soft connective tissue volume (mm^3) and bone to total volume ratio. Bone / total volume ratio; It is found by proportioning the bone volume remaining within the boundaries determined by the tissue to the volume of all other structures (connective tissue and cavities) (4). Bone and soft connective tissue volumes and bone / total volume ratio results enabled us to comment on bone tissue in the region we examined.

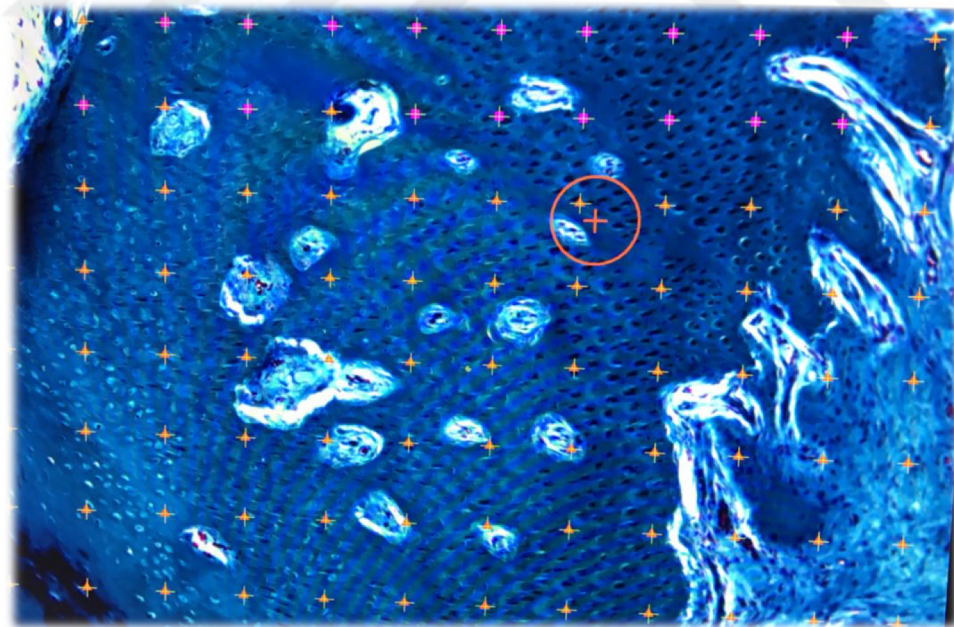


Fig. 3.12. Midpalatal suture area point counting

3.3.2. Blood Samples and Obtaining Blood Serum Plasma

Following the 10 and 20 days retention time, rats were sacrificed with guillotine under diethyleter anesthesia and blood samples of 2-2,5 ml were collected. Blood was drawn into tubes and samples are kept at 4C° for 1 hour. Blood serum was obtained by centrifugation for 20 minutes at 3000 rpm. Blood serum was separated from erythrocytes and white blood cells. Blood serums were transferred in 75 µl aliquots and stored at -80 C until analysis. For each experiment, sub-aliquoted serum samples were thawed, used for the experiment, and discarded. Each aliquot was discarded after one use so that each aliquot of serum was thawed only once for 5 each experiment, so no sample was repeatedly frozen and thawed. This allowed us to eliminate any possible effects of repeated freezing and thawing on the serum integrity.

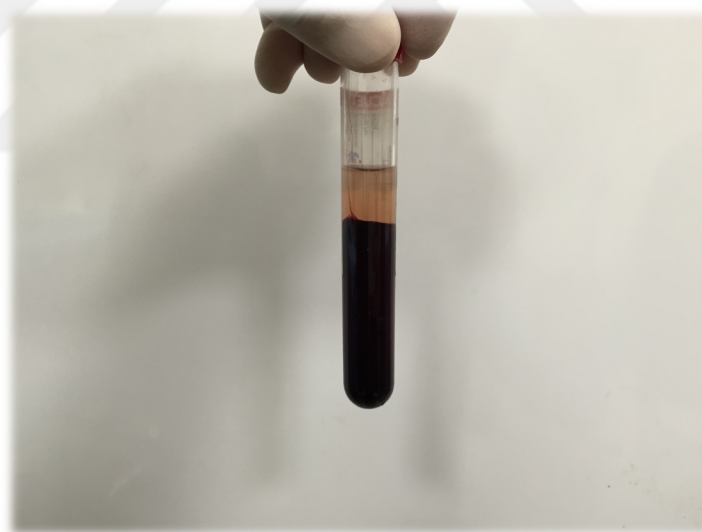


Fig. 3.13. Obtaining blood serum plasma

3.3.2.1. Biochemical Analysis

Commercial Blood Serum Hyaluronic Acid Level Indicator Set (Shanghai Yehua Biological Technology, Shanghai, China) was used to indicate blood serum HA level. The working procedures recommended in the package insert in the kits were applied. Double measurements were made from each sample, and the lowest and highest level measured samples were re-analyzed and averaged. This kit uses enzyme-linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay rat hyaluronic acid (HA). Hyaluronic acid (HA) was added to wells that are pre-coated with hyaluronic acid (HA) monoclonal antibody and then incubated. After incubation, anti HA antibodies labeled with biotin were added to unite with streptavidin-HRP, which forms the immune complex. Unbound enzymes were removed after incubation and washed, then substrate A and B were added. The solution has turned blue and changed to yellow with the effect of acid. The shades of solution and the concentration of rat hyaluronic acid (HA) were positively correlated.



Fig. 3.14. Blood serum hyaluronic acid level indicator set (Shanghai Yehua Biological Technology, Shanghai, China)



Prepare reagents, samples and standards.



Add prepared samples and standards together with second antibody labeled with biotin and ELISA solutions. Allow reaction for 60 minutes at 37°C.



Wash the plate five times. Add chromogen reagent A and B. Incubate for 10 minutes at 37 °C for color development.



Add stop solution



Read the absorbance (OD) value within 10minutes.



Calculate.

Fig. 3.15. blood serum hyaluronic acid level indicator set application procedure.

3.4. Statistical Evaluation

The statistical analysis of the histological and clinical results were made using Statistical Package for The Social Sciences version 20.0 software (SPSS Inc., Chicago, IL, USA). In each group, descriptive mean and standard deviation of each parameter were determined. One-way analysis of variance (ANOVA) test, Mann Whitney U test and Kruskal Wallis test was used for the comparison of parameters between the groups. Post hoc Tukey HSD tests were performed to compare 2 groups of variables. A significance level of $P < 0.05$ was used for all statistical comparisons. The results were evaluated in confidence interval of 95%.



4. RESULTS

4.1. Observational Results

There is no any soft tissue healing problem in injection area in all groups. Also, no signs of inflammation was observed in all groups.

When we look at the weight loss, significant differences were observed between control, HMWHA and LMWHA groups on days 5, 10 and 20.

Table 5. Weight loss in control and experiment group animals

		<i>T1</i>	<i>T2</i>	<i>T3</i>	<i>p</i>
CG10	<i>Ort±Ss</i> <i>Min-Max</i> <i>(Median)</i>	355±32,84 310-400 (347,5)	315,63±31,67 270-365 (317,5)	334,25±21,29 290-355 (337,5)	0,003**
CG20	<i>Ort±Ss</i> <i>Min-Max</i> <i>(Median)</i>	383,13±49,71 300-430 (397,5)	356,25±45,57 280-400 (370)	369,38±42,55 290-430 (375)	0,003**
HMWHA10	<i>Ort±Ss</i> <i>Min-Max</i> <i>(Median)</i>	362,5±34,02 320-410 (347,5)	315,63±32,78 280-355 (305)	320,63±26,65 265-345 (325)	0,002**
HMWHA20	<i>Ort±Ss</i> <i>Min-Max</i> <i>(Median)</i>	362,5±43,59 285-420 (375)	339,25±30,76 280-370 (344,5)	352,75±26,17 325-405 (353,5)	0,044*
LMWHA10	<i>Ort±Ss</i> <i>Min-Max</i> <i>(Median)</i>	345±45,83 285-415 (340)	318,5±32,48 265-365 (322,5)	322,13±30,1 265-360 (328,5)	0,024*
LMWHA20	<i>Ort±Ss</i> <i>Min-Max</i> <i>(Median)</i>	353,75±56,05 305-470 (335)	340±53,05 280-435 (317,5)	355,63±68,27 250-445 (342,5)	0,135

CG-10 value shows statistically significant difference according to the periods ($p=0.003$; $p<0.01$). The fact that the CG-10 value in the T1 period was higher than the T2 period was found to be statistically significant ($p=0.001$; $p<0.01$).

The CG-20 value shows statistically significant difference according to the periods ($p=0.003$; $p<0.01$). The fact that the CG-20 value in the T1 period was higher than the T2 period was found to be statistically significant ($p=0.001$; $p<0.01$).

The HMWHA-10 value shows a statistically significant difference according to the periods ($p=0.002$; $p<0.01$). The fact that the HMWHA-10 value in the T1 period was higher than the T2 and T3 values was found to be statistically significant ($p=0.001$; $p<0.01$).

HMWHA-20 value shows statistically significant difference according to the periods ($p=0.044$; $p<0.05$). The fact that the HMWHA-20 value in the T2 period was lower than in the T3 period was found to be statistically significant ($p=0.001$; $p<0.01$).

LMWHA-10 value differs statistically significantly according to the periods ($p=0.024$; $p<0.05$). It was found statistically significant that the LMWHA-10 value in the T1 period was higher than in the T2 period ($p=0.001$; $p<0.01$).

LMWHA-20 value does not show a statistically significant difference according to the periods ($p>0.05$).

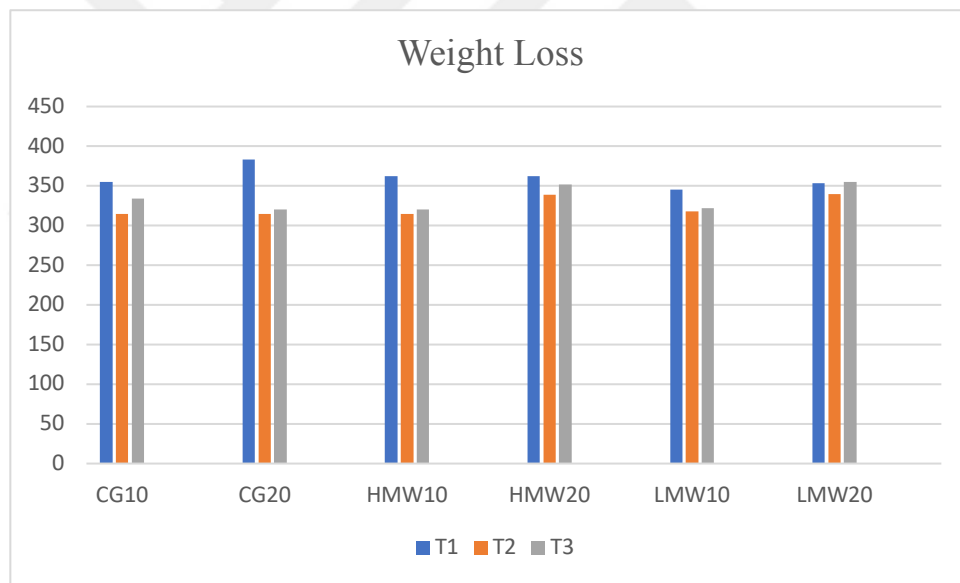


Fig. 4.1. Weight loss comparisons on days 5, 10 and 20 among the groups

4.2.Histomorphometrical Findings

4.2.1.Bone Volume (mm³)

Table 6. Bone volume levels and comparison of all groups

Group Name	(Bone Volume $\mu\text{m}^3 \pm \text{Standart Deviation}$) n=5	p
CG10 & HMWHA10	40568000000 $\pm$ 247159528,531 & 67842900000 $\pm$ 1791809163,946	0,000
CG10 & LMWHA10	40568000000 $\pm$ 1791809163,946 & 59271700000 $\pm$ 1496705202,548	0,000
CG10 & KK20	40568000000 $\pm$ 247159528,531 & 46604400000 $\pm$ 1791809163,946	0,251
HMWHA10 & HMWHA20	67842900000 $\pm$ 1791809163,946 & 64678260000 $\pm$ 1726439069,183	0,833
LMWHA10 & LMWHA20	59271700000 $\pm$ 1496705202,548 & 59448400000 $\pm$ 2003284150,256	0,391
LMWHA10 & HMWHA10	59271700000 $\pm$ 1496705202,548 & 67842900000 $\pm$ 1863013446,901	0,044
CG20 & HMWHA20	46604400000 $\pm$ 1791809163,946 & 64678260000 $\pm$ 1726439069,183	0,000
CG20 & LMWHA20	46604400000 $\pm$ 1791809163,946 & 59448400000 $\pm$ 2003284150,256	0,001
LMWHA20 & HMWHA20	59448400000 $\pm$ 2003284150,256 & 64678260000 $\pm$ 1726439069,183	0,050

As a result of comparing the average bone volume amounts between 10 and 20 day same groups;

- There is no significant result between 10 days control group and 20 days control group (0,251).
- When the 10 days LMWHA group is compared with the 20 days LMWHA group, there is no significant result (p=0.391)
- Again, no significant result is found in the comparison of 10 days HMWHA and 20 days HMWHA group (p=0.833).

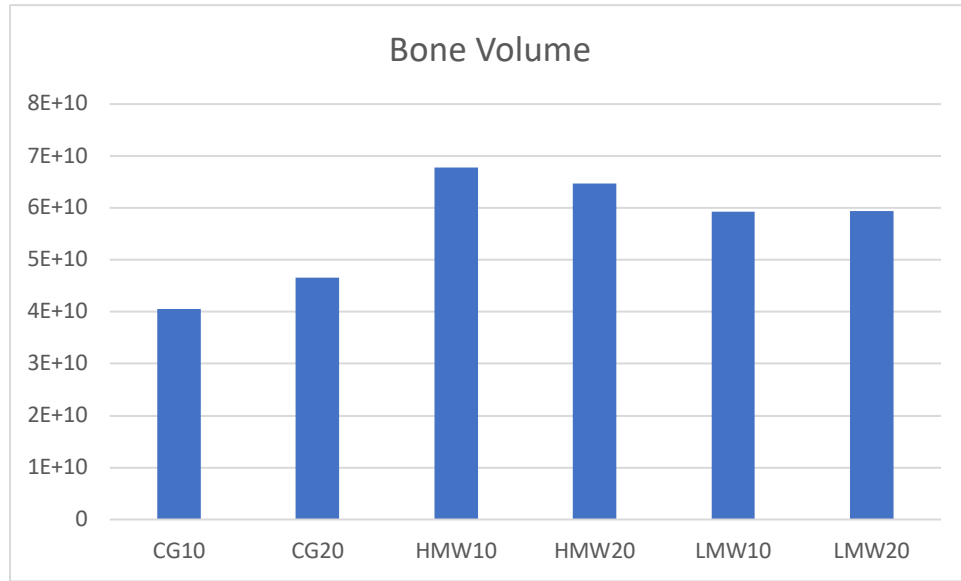


Fig. 4.2. Bone volume levels for all groups
 CG10 and CG20 (Control Group 10 and 20 days)
 HMW10 and HMW20 (Healon GV OVD 10 and 20 days)
 LMW10 and LMW20 (AMO VITRAX OVD 10 and 20 days)

4.2.1.1. Comparison of 10 and 20 Days Experiment Groups Separately

Table 7. Comparison of bone volume of 10 days experiment groups

Group Name	(Bone Volume $\mu\text{m}^3 \pm$ Standart Deviation) n=5	p
CG10 & HMWHA10	40568000000 $\pm$ 247159528,531 & 67842900000 $\pm$ 1791809163,946	0,000
CG10 & LMWHA10	40568000000 $\pm$ 1791809163,946 & 59271700000 $\pm$ 1496705202,548	0,000
LMWHA10 & HMWHA10	59271700000 $\pm$ 1496705202,548 & 67842900000 $\pm$ 1863013446,901	0,044

As a result of comparing the average bone volume between the experimental groups;

In the 10 day experimental groups, the control group was significantly lower than the HMWHA 10 group ($p = 0,000$)

When the control group and the LMWHA 10 group are compared, it is seen that the control is significantly lower than LMWHA 10 ($p = 0.000$).

When comparing the LMWHA 10 and HMWHA 10 groups among themselves, the bone volume in the LMWHA 10 group is found lower HMWHA 10 group ($p = 0.044$).

In line with these results, it can be said that the effect of LMWHA 10 AMO VITRAX OVD substance compared to HMWHA 10 HEALON GV OVD substance is less effective in the formation of new bone.

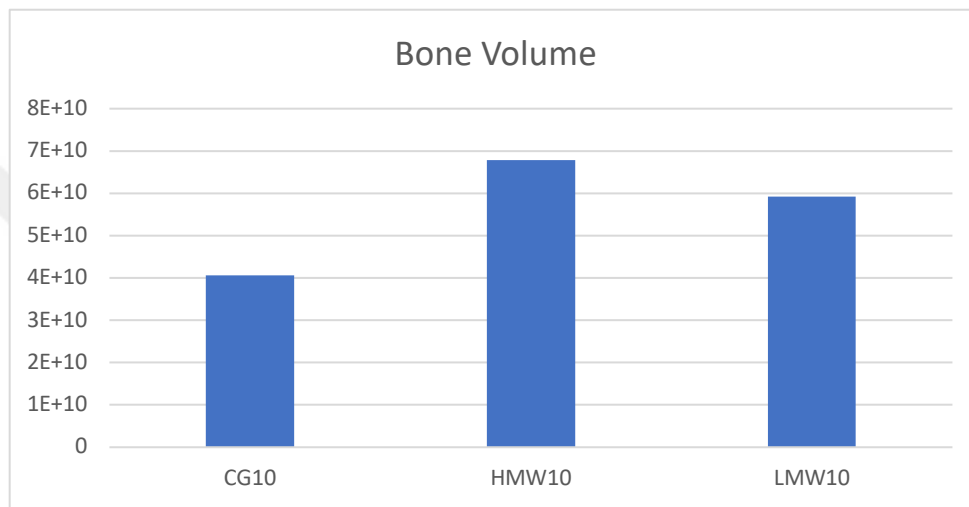


Fig. 4.3. Bone volume levels of 10 days group

Tablo 8. Comparison of bone volumes of 20 days experimental groups

Group Name	(Bone Volume $\mu\text{m}^3 \pm \text{Standart Deviation}$) n=5	p
CG20 & HMWHA20	$46604400000 \pm 1791809163,946$ & $64678260000 \pm 1726439069,183$	0,000
CG20 & LMWHA20	$46604400000 \pm 1791809163,946$ & $59448400000 \pm 2003284150,256$	0,001
LMWHA20 & HMWHA20	$59448400000 \pm 2003284150,256$ & $64678260000 \pm 1726439069,183$	0,050

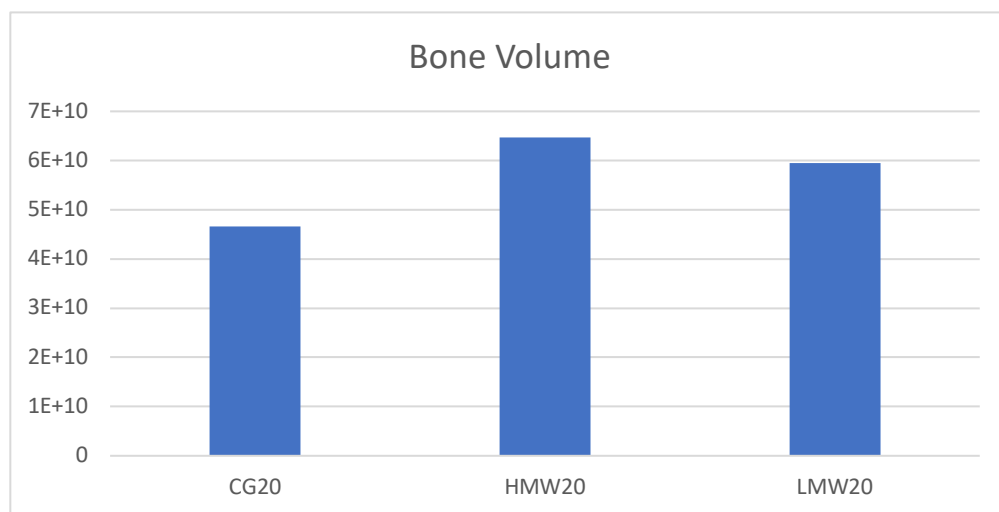
Comparison of bone volumes of 20 day experimental groups;

In the 20 day experimental groups, the control group is significantly lower than the HMWHA 20 group ($p = 0,000$)

When the control group and the LMWHA 20 group are compared, it is seen that the control is significantly lower than this ($p = 0.001$).

When comparing the LMWHA 20 and HMWHA 20 groups among themselves, the bone volume in the LMWHA 20 group is found lower than HMWHA 20 ($p = 0.050$).

Considering these 20 day results, depending on the duration of this experiment, LMWHA 20 had an effect similar to the 10day duration of the test compared to the HMWHA 20.

**Fig. 4.4. Bone volume levels of 20 days group**

4.2.2. Soft Tissue Volume (micrometer³)

Table 9. Soft tissue volume levels and comparison of all groups

Group Name	(Average Soft Tissue Volume $\mu\text{m}^3 \pm \text{Standart Deviation}$) n=5	p
CG10 & HMWHA10	7200890000 $\pm$ 166407556,21866 & 12733400000 $\pm$ 529677650,40006	0,001**
CG10 & LMWHA10	7200890000 $\pm$ 166407556,21866 & 6943000000 $\pm$ 1065960904,53637	0,001**
CG10 & CG20	7200890000 $\pm$ 166407556,21866 & 8041810000, $\pm$ 450506732,58010	0,924
HMWHA10 & HMWHA20	12733400000 $\pm$ 529677650,40006 & 9965760000 $\pm$ 684412381,73098	0,055
LMWHA10 & LMWHA20	6943000000 $\pm$ 1065960904,53637 & 5602500000 $\pm$ 413929506,88090	0,997
LMWHA10 & HMWHA10	6943000000 $\pm$ 1065960904,53637 & 12733400000 $\pm$ 529677650,40006	0,001**
CG20 & HMWHA20	8041810000, $\pm$ 450506732,58010 & 9965760000 $\pm$ 684412381,73098	0,283
CG20 & LMWHA20	8041810000, $\pm$ 450506732,58010 & 5602500000 $\pm$ 413929506,88090	0,104

Mann Whitney U Test * $p < 0,05$ ** $p < 0,01$

As a result of comparing the average soft tissue volume amounts between experimental groups;

Soft tissue volume value of the CG10 group is found to be lower than the HMWHA 10 group, which is statistically significant ($p=0.001$; $p<0.01$).

Average soft tissue value of the CG10 group is found to be higher than the LMWHA10 group, which is statistically significant ($p=0.001$; $p<0.01$).

When comparing the LMWHA10 and HMWHA10 , soft tissue level in HMWHA10 group is found significantly higher than LMWHA10 group ($p=0.049$; $p<0.05$).

The mean soft tissue volume value does not show a statistically significant difference between the CG10 and CG20 groups ($p>0.05$). Also there is no significant difference between HMWHA10 and HMWHA20 groups ($p>0.05$). Same with others there is no statistically significant difference between LMWHA10 and LMWHA20 groups ($p>0.05$).

Soft tissue volume value of the LMWHA10 group is found to be lower than the HMWHA10 group ($p=0.001$; $p<0.01$). There is no statistically significant difference between the CG20 and HMW20 groups ($p>0.05$). The mean soft tissue volume value does not show a statistically significant difference between the CG20 and LMWHA20 groups ($p>0.05$).

When we compare 20 days groups; there is no statistically significant difference between CG20 and HMW20 also CG20 and LMWHA20 groups ($p>0.05$). But soft tissue volume of LMWHA20 group is found to be lower than HMW20 group, which is statistically significant ($p>0.05$).

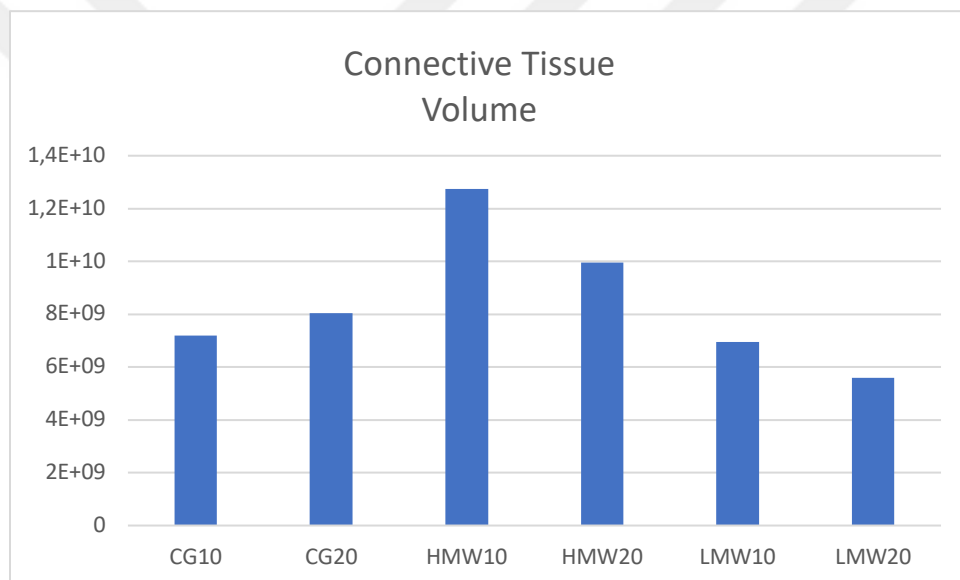


Fig. 4.5. Soft tissue volume levels for all groups
 CG10 and CG20 (Control Group 10 and 20 days)
 HMW10 and HMW20 (Healon GV OVD 10 and 20 days)
 LMW10 and LMW20 (AMO VITRAX OVD 10 and 20 days)

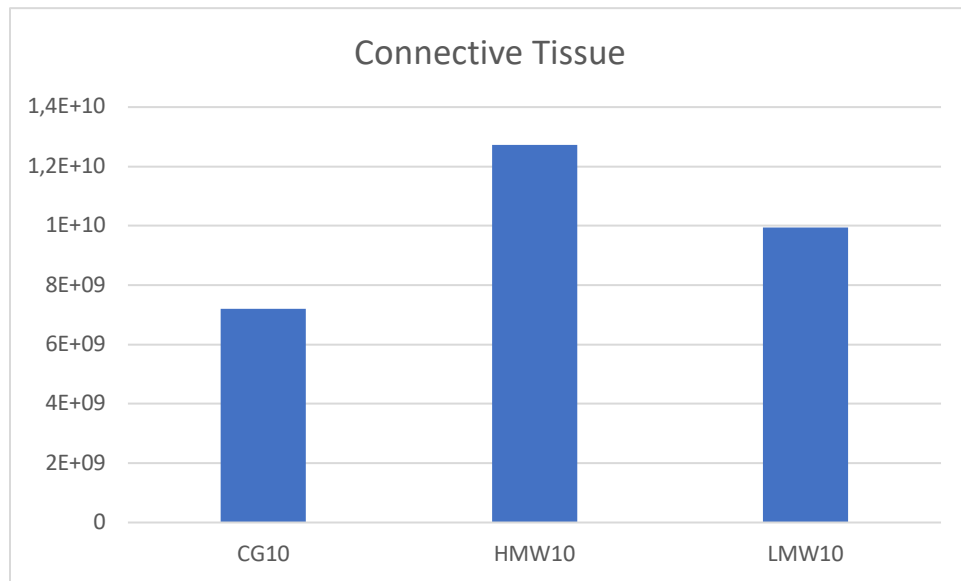


Fig. 4.6. Soft tissue volume levels of 10 days group

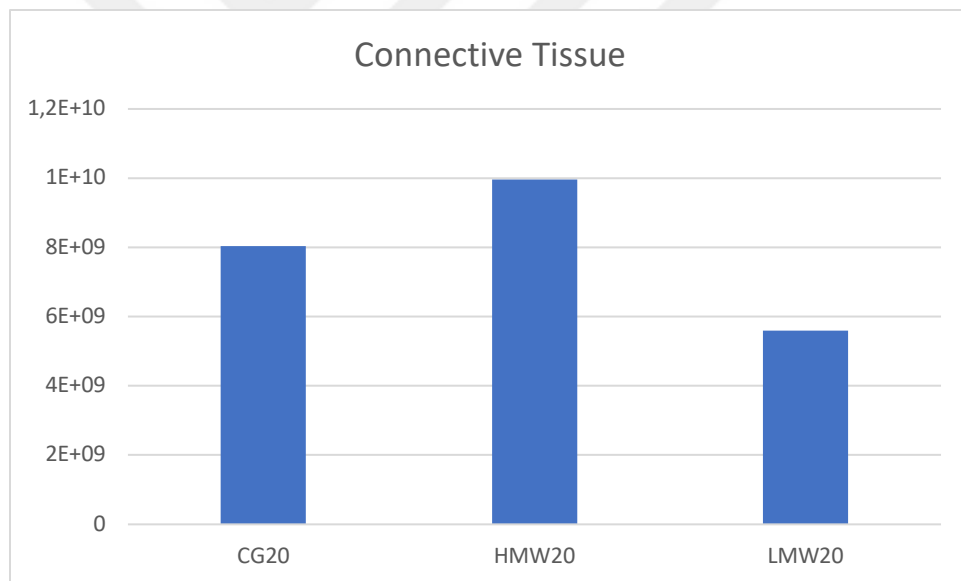


Fig. 4.7. Soft tissue volume levels of 20 days group

4.3. Biochemical Results

Table 10. Average blood plasma HA level and standart deviation of all groups

Group Name	(Average Blood Plasma Serum HA Level $\mu\text{m}^3 \pm \text{Standart Deviation}$) n=5	p
CG10	306,29 $\pm$ 39,94	0,149
CG20	319,03 $\pm$ 29,55	0,149
HMWHA10	334,91 $\pm$ 33,03	0,149
HMWHA20	334,74 $\pm$ 32,26	0,149
LMWHA10	352,22 $\pm$ 22,06	0,149
LMWHA20	318,86 $\pm$ 26,69	0,149

Kruskall Wallis Test * $p < 0,05$ ** $p < 0,01$

When we look at biochemical analysis;

There is no significant difference observed between all groups ($p > 0,05$). This result can be explained by the half life of HA.

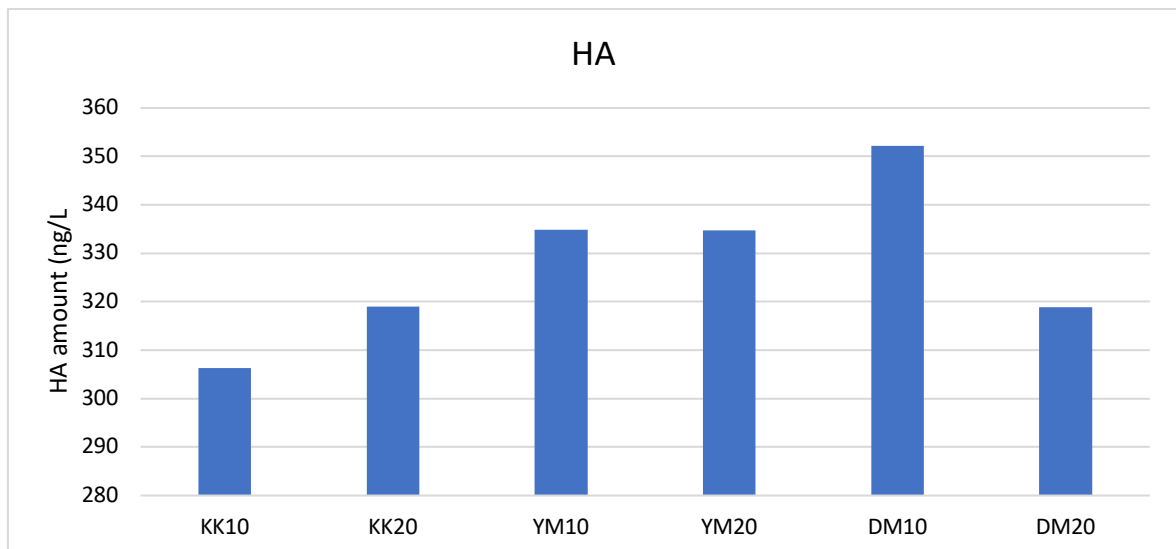


Fig. 4.8. Blood plasma serum HA levels for all groups
CG10 and CG20 (Control Group 10 and 20 days)
HMW10 and HMW20 (Healon GV OVD 10 and 20 days)
LMW10 and LMW20 (AMO VITRAX OVD 10 and 20 days)

5.DISCUSSION

The aim of our study is to investigate the local effects of different molecular weight HA on the amount of bone formation after maxillary expansion and also to observe the systemic effects in blood serum at short and long time periods. In this experiment we used HA, which is a polysaccharide content of ECM, in two forms: HMW (Healon GV®) which has an adhesive structure and LMW (AMO® Vitrax II), which has a diffuse structure. In our study, both forms of HA were applied locally and their local and systemic effects were examined histomorphometrically and biochemically.

With this study, we aim to increase rate of bone formation in short time and reduce the risk of relapse after RME thus to eliminate or reduce the time of retention procedure, which requires appliances that are not easy to accept by the patients. Schauseil et al (217) investigated CBCT's of rapid maxillary expansion patients after six months retention period. Even after six months and more retention period CBCT results show %50-70 bone healing. Same with this results, De Mole (218) and Gurgel (219) worked on adults and mixed dentition patients and found the same CBCT and radiographic results between 3-4 months retention periods.

In our study, rapid maxillary expansion was applied to the upper jaws of rats. This is a frequently used method to treat transversal direction anomalies in humans (10,19,49,50,125). After RME, fracture areas and bone islets are formed in the midpalatal suture. The healing of the midpalatal suture is basically the fusion of the bone islets between the segments (121).

In our study, we used HA, because there are only a few studies in the literature about its effects on the midpalatal suture (217). HA is known for its accelerating effect on bone and cartilage formation and is used in many different types of human and animal studies. HA is located in active cell growth sites and has a role in cell growth by binding to receptors in divisible cells (154). All the known effects of HA considered, some studies (175,177) have researched that HA can accelerate the bone formation on different body sections. Accelerated bone formation in the midpalatal suture area can reduce the time of skeletal retention after RME. In addition to that we had the hypothesis that short term and long term

effects of LMW and HMW HA can bring out different results in the bone amount. We also studied the systemic effects of LMW and HMW HA by making biochemical investigation in blood serum.

We compared the effects of HMW and LMW HA on bone formation in our study. HA is known to have different molecular weights vary between 1×10^5 and 10^8 Da (145,158,159). There are studies (136,161-169) and systematic reviews (170,171) that compare the effects of HMW and LMW HA on bone and cartilage tissue. There are also studies that evaluate only the HMW HA (139,158,173-181,186) and only the LMW HA (138,167,182,187). Although the efficacies of HMW and LMW HA were examined separately in these studies, the importance of molecular weight, viscosity and density is still not clear. HA with HMW and LMW is usually related to their effect on joint areas, but there are still not enough studies about the effects on bone healing. In order to overcome this deficiency, application of two different HA substances on bone tissue in the midpalatal suture area is planned. The rats were sacrificed in two different time periods to evaluate the short and long term local effects of HMW and LMW HA on bone tissue and blood serum plasma HA level evaluated.

In our study we preferred rats, which are one of the smallest animal models. Monkey (230,231), cat (232), rabbit (127,177), pig (233), mouse (234) and rat (123,125,228) were the animals that had been used in studies about the expansion of sutures.

Rats are often preferred in scientific studies because of their ease of reproducibility, keeping and caring, low costs, and histological preparation process. The histology of the sutural and bone changes under stress can be best observed in rabbits and rats (219). Even though the mechanisms look alike in humans and rats following orthodontic force application, partial differences are present. Soylu et al (235) stated that alveolar bone of the rats lack bone marrow spaces and osteons. Calcium metabolism is controlled by intestinal absorption rather than the bone tissue itself. When all of these come together, it can be concluded that the bone density of the rats is higher than that of humans. Remodelling activity is also faster than that of humans.

The amount of expansion in the suture area depends on the age and metabolic rate. In order to avoid these differences, all rats were chosen 10-12 weeks of age and had similar weights in our study. Misawa et al. (227), researched the relationship between the age and

rate of alveolar bone construction and destruction in rats. They stated that, under physiological conditions, the rate decreased significantly between 6 week old and 30-40 week old rats. As the rats aged, the bone metabolism continued to decrease further. Andreollo et al. (221) reported in their study, that a day of a rat's life corresponds to 30 human life days. Other researchers have found similar results and reported that a male rat weighing 180-200 grams corresponds to a male individual around age of 14, who is still in the development of growth period (125).

In order to eliminate the changes related to estrous cycle, we preferred male rats in our study. Likewise, all animals were selected from the same breed in order to prevent changes that may result from genetic differences.

As in the study of Aslan (236), due to rapid absorption and ease in application, the animals were anesthetized intraperitoneally at the beginning of the experiment prior to orthodontic force application. Accordingly, following injection of 10% Ketazol® (60 mg/kg/im, including 100 mg/ml ketamine hydrochloride, Richter Pharma AG, Wels, Austria), 2% Rompun® (5 mg/kg/im, including 23,32 mg/ml xylazine, Bayer HealthCare LLC., Cansas, USA) was injected to relax the muscles and avoid spontaneous movements during the intervention.

For maxillary expansion of the rats, we used a helix design appliance from 0,014 inch stainless steel wire. 50 grams of force was applied for 5 days. Burstone and Schafer (228) stated that young rats have a thickness of around 20-60 µm in the midpalatal suture area and 5 days of expansion can create around 377 ± 104 µm opening in the suture area.

As a result of literature research, the techniques of Uysal et al. (123,237-240) were used in our experiment. Unlike these studies, the appliance was placed on the palatal mucosal sides of the upper incisors in order to prevent rats from interfering with their paws and to be closer to the suture area. In similar studies (124,241), the effects of different substance injections following maxillary expansion were examined. Similar to our study, Uysal et al. (123,237-240) applied 30-50 grams of force with 0.014 inch thick single helix stainless steel wire for 5 days of expansion. There are different techniques among the applied expansion

methods. The common mechanisms of different application techniques are based on the principle of separation of suture under pressure, creating an orthopedic effect.

In the studies conducted by the researchers regarding the activation and retention phase, which lasted for 15 days from the first day of the expansion process, the 14-day bone healing period in rats was found sufficient for differentiation and proliferation of cells (123,237-240).

The rats' alveolar bones are more dense and contain less marrow cavity than humans. Following the application of an orthodontic force the resulting reaction in humans is seen around in 30-40 hours while in rats within 6 hours and the basic mechanisms are similar (225). Besides, due to differences in bone structure of rats, cementoblast cells react faster than osteoblast cells and perform remodelling faster (226). Li et al. (229) reported that fracture healing was completed in 5-week period and the fastest bone formation occurs within 3 weeks in rats. Because of this reason in the present study we plan to investigate two different retention period : 10 and 20 days.

In order to avoid losing the space after expansion, active helix device was removed and a passive retention wire was inserted into the incisors of rats for 10 and 20 days. Retention devices are used to prevent relapse after orthodontic treatment. Following the expansion in the midpalatal suture area, it has been reported that the reconstruction of hard tissues is beginning (69,75,230,242).

The sections obtained in our study were histomorphometrically evaluated with the program Cavalieri estimator of Stereoinvestigator 11.0 (Microbrightfield, Williston, VT, ABD). This kind of image analysis programs enabled us to measure the volume and length values of the histological data. Histomorphometry is a frequently used method in quantitative examination of changes occurring at the cellular level (123,237-240,243).

In our study, according to the results of the effects of HA with LMW and HMW applied to midpalatal suture area following rapid maxillary expansion, new bone formation has increased in the all experiment groups LMWHA 10, LMWHA 20, HMWHA 10 and HMWHA 20. It was observed relatively high osteoblast and capillary cell values in all

experimental groups in the midpalatal suture area and its surroundings comparing the control groups. Blood serum HA level is the highest at LMWHA groups but there is no any significant differences. In parallel with our study, Iwata et al. (136) found out that density, molecular weight and viscosity of HA level decrease in damaged tissue. HMWHA has the highest level of biological function while its structure is intensely polymerized and they found that LMW HA increase vascular activity in tissues.

Similar to our findings, in-vitro studies (173,178,179,180) showed that HMWHA helps to agglomerate isolated proteoglycans by creating a gag in wound formation and increase the formation of sulfated GAG synthesis. When HA was applied externally in the culture medium, it stimulated HA formation and fibroblasts in tissue. It has been shown to prevent the transport of leukocytes into tissues and the release of enzymes by phagocytosis by IgG and neutrophils. In other studies conducted in in-vitro environment, it has been stated that mesenchymal progenitor cells undergo cell proliferation by adhering to the skeletal structure formed by HA (244,245).

Supporting that the osteoblast enhancing effect of the HA that we apply may be due to MW, Huang et al. (246) stated that HA, which has a triggering effect on osteoblast cells, can directly or indirectly improve bone-forming properties of bone grafts, depending on the MW and administered dose. Liguori et al.(247) has reported that HA increases the formation of fibrin during the wound healing process, activates the phagocytic activities of neutrophil and macrophage cells, increases the release of chemotactic factors, and increases the collagen fibers in the granulation stage.

According to ESCISIT 2003 report, which prepared the recommendations of the European League Against Rheumatism (EULAR) and examines the effectiveness of different MW HAs in knee osteoarthritis, it is recommended to prefer HMWHA (165). Smith et al. (166) examined HA molecules with MA ranging from 75,000 to 4,7 MDa in-vitro and found that the addition of HMWHA applied to the cell culture increased HA synthesis in the tissue. Tammi et al. (158) stated that HA acts as a filter for macromolecules thanks to its HMW structure and is able to bind to cell surface proteins and other matrix proteins.

Lo et al. (170) examined the effects of HA with 7 different MWs on OA. Accordingly, HA with HMW preparations are more effective than HA preparations with LMW, but they

claimed that the results are hard to interpret because of the heterogeneous distribution between studies.

Wobig et al. (248) examined the efficacy and elascosceous effects of HA's HMW and LMW derivatives on OA. They reported that the HMW derivate (Hylan® G-F 20), which was applied locally to joint areas of OA patients, has a higher elascoviscosity than LMW derivates and is more effective in terms of relieving the joint pain.

Mendes et al (175) examined the effects of HMWHA on bone tissue by extracting the molar teeth of the rats and filling one of the extraction cavities with 0,1 ml 1% HMWHA and the other one on the same rat with blood clot. They observed the histomorphological changes at the end of 2nd, 7th and 21st days. At the end of the 7th day, new bone formation, bone trabeculae, and blood vessel density were found significantly higher in HMWHA group than the control group. Their findings were in agreement with our study, which proves that HMW HA increases osteoblastic activity and capillary cell amount when it is applied to the bone.

Similar to our study but different in terms of follow-up times and choice of rabbits as experimental animals instead of rats, Aslan et al. (177) applied HMWHA (Healon®, Pharmacia & Upjohn, Sweden) to the bone cavities they formed in tibia. They applied only HMWHA to one group and HA mixed with bone grafts to the other group and examined their effects on the 20th, 30th and 40th days. According to the results of their studies, they achieved higher bone values in the groups that underwent bone grafting with HA on the 30th and 40th days compared to the group treated with HA alone. In both experimental groups, they obtained statistically significantly higher bone values compared to the control groups. HA and bone grafts are known for their bone formation accelerating effects. Although there are differences with our study in terms of follow-up times and testing in rabbits, HMWHA increased bone formation. In our study, when the HMWHA applied to the midpalatal suture area was applied alone, increased osteoblastic activity was observed.

In our study, it was found that osteoblastic activity was high in the locally HMWHA applied group. Stancikova et al.(162) investigated the effects of different MW HAs on rats

and they obtained similar findings to our study. They administered LMWHA (0.75 MDa) and HMWHA (1.62 MDa) orally to 1mg / kg / day and 0.5mg / kg / day to rats that underwent overectomy and studied the effects of HA. They reported that bone destruction after overectomy was prevented in groups where HMWHA was applied 1mg / kg / day, and bone mineral density was increased and bone density was maintained. In our study, unlike the study of Stancikova et al. (162), HA was applied locally to the midpalatal suture area and similar results were obtained.

In our study, significant differences were observed between the control group and the HMWHA group in terms of bone formation. Similarly, Baldini et al. (181) in their studies on humans, filled the dental extraction sockets of the experimental groups by mixing HMWHA (> 5MDa) with the autologous bone graft, and as the control group they filled the other tooth extraction cavity in the same jaw only with the autologous bone graft. After a 4-month follow-up period, they took a biopsy from these areas and evaluated the sections histologically, and the findings clinically and radiographically. In histological sections and as a result of the radiographic evaluation they received, they reported that they saw rich bone trabeculae and mature bone cells in the experimental group and that they could not get dense bone images in the control group. They reported that HMWHA had no bacterial spread and signs of infection due to the bacteriostatic effect. As Toole (230) reported in his studies examining the effects of HA, HA has increased multiple factors in tissue morphogenesis, cell migration, cell differentiation and adhesion stages by communicating with macromolecules thanks to its physical, chemical properties and biological structure. Although there were differences in the duration of follow-up, applied area and application in humans from the study of Baldini et al. (181), HMWHA showed positive results on bone formation in both studies.

Similar to our study in terms of our application in rat bones, in their study in 2002 Zanchetta et al. (138) filled the 5 mm bone cavity they formed in the parietal bones of rats with 1mg HA derivative HE 800 (800 kDa) produced from bacterial exopolysaccharide. As the control group, they filled the other parietal bone cavity of the same rat with collagen. In the sections that they evaluated radiographically and histologically at the end of 15 days, the bone cavities of the experimental groups were filled with dense bone cells at the rate of $95.9 \pm 6.2\%$, and this rate was $17.8 \pm 18.1\%$ in the control groups.

In parallel with our study in terms of HA being applied in bone tissue, in the study of Engstrom et al. (174) in 15 patients, the Rooster component with HMWHA was applied locally after surgical ($> 6\text{mm}$) and non-surgical ($> 5\text{mm}$) interventions on teeth with periodontal bone damage. HA mixed with the membrane after surgical intervention was placed in the area with periodontal bone damage. In non-surgical intervention, HA was applied for 3 weeks, once a week. They reported that after 12 months of follow-up clinical findings, the group who underwent HA had a significant decrease in periodontal pocket depths compared to the group without HA, while the amount of increase in bone level was less than 1mm in radiographic findings.

Similar to our study in terms of HA's application to bone tissue, the injected solution being in similar MWs and the results obtained from the HMWHA group, Sasaki and Watanabe (176) applied HMWHA (1900 kDa) locally to the bone cavities they created in the rats femoral area. They examined ossification for 14 days and reported that as of day 4, new bone formation began in the HA group, on the cavity base and walls, at the end of the first week, the cavity was completely filled with trabecular bone, and the granulation tissue formed in the first period quickly replaced the new bone marrow tissue. In the control group, the newly formed bone tissue began to appear at the end of the 7th day, and at the end of the 14th day it was completely filled with trabecular bone. These results suggest that HMW HA is capable of accelerating bone tissue formation through mesenchymal cell differentiation in bone wounds.

Similar to our findings and the fact that HA was applied in bone tissue, Zanchetta et al. (183), formed 5mm cavity areas in the parietal bones of rats. They injected 0.5 mg HE 800 (800 kDa), which is a HMWHA derivate, leaving the cavities in the control groups empty. According to the findings of the study, they reported that both cavities were completely filled with bone at the end of 21 days, and that this effect in the control groups may have been due to the systemic effect of HA. In the study of Sasaki and Watanabe (176), it may be thought that bone development may have been indirectly affected in the control groups due to the systemic effect of HA, or that the difference between the follow-up periods of our and their studies may have affected the findings.

In contrast to the findings of our study, Pilloni and Bernard (168) reported in their *in-vitro* study, that LMWHA (30 kDa) increased the formation of bone by providing

mesenchymal cell migration and differentiation whereas HMW HA was less effective in bone formation due to its viscosity values. Similarly, Deed et al. (169) stated in their in-vitro study that HMW HA has a decreasing effect on the vascularization rate in tissues, and that fragmented forms of HA increases vascularity and endothelial cell proliferation in tissues.

Sasaki and Watanabe (176) reported that HMWHA had positive effects on the formation of new bone in the wound healing process, but it was not known whether HA's effect on bone-forming cells was directly or indirectly.

Different from the results of Pilloni and Bernard (168) and Deed et al. (169), in parallel with Sasaki and Watanabe (176), HMWHA, which we applied in our study, is found in polysaccharide and monosaccharide forms in in-vivo environment and it accelerates healing processes in tissues. It can be thought to be effective in their time. In addition, it can be thought that the in-vitro examination of the studies (168,169) may not have reflected the changes in living tissues completely and thus caused the difference in the findings.

Unlike the findings of our study, in the studies of Ballini et al. (182) used a HA preparation similar to the LMWHA solution we use, the material of LMWHA (Hyaloss®) in esterified form in patients with periodontal bone destruction (8.3mm). They mixed the HA with autologous bone graft and examined the effects of these substances on bone formation. They evaluated the results with 10-day and 6, 9, and 24-month radiographic follow-ups, and stated that the gingivitis ratio was between 0-1 in 10-day controls due to the bacteriostatic effect of HA, but they did not see bone remodeling in this process. They reported that bone cavities filled very well with moderate bone remodeling at 6-month radiographic controls, and they measured periodontal pocket depth measured as 2.6 mm in 9-month controls. At the end of 24 months, they stated that they achieved the desired results with their fully filled bone cavities.

If we compare the results of the 10th and 20th day in terms of the difference between the findings of the study of Ballini et al. (182) with our study, LMWHA does not show intense bone remodeling as HMWHA. Based on this study, it can be thought that by reaching the desired bone level at the end of 24 months, the effectiveness of LMWHA may be slow and / or mixing LMWHA with bone graft may have increased the effect of this molecule on bone remodeling.

Unlike our findings, in the study of Asari et al. (167) on dogs, LMWHA (840.000 Da) showed higher permeability to synovial membrane than HMWHA (2.7 MDa). They stated that the only factor in increasing the synthesis of HA was not MW and there was no human study on this subject. In the review article of Arrich et al. (171) investigating the effects of HA on joints with OA, there was no difference in the effects of different MW HA preparations. It may be thought that the reason for the difference between our findings and the different researches may have been due to the structural differences of cartilage and bone tissues and the heterogeneous distribution of studies in-vitro, in-vivo and animal experiments.

Similarly to our study, Bingöl et al. (161) compared two different MW HAs and examined the effects and side effects of Methyl Prednisolone acetate preparation containing corticosteroids by applying intraarticular injection to the knee joints of humans. They evaluated their findings at the end of the 2nd and 6th months after the injection. They applied intraarticular injection 3 times with one week intervals with LMW HA (Hyalgan® 500-730 kDa MA, 20 mg / 2ml) and HMW HA (Orthovisc® > 1MDa MA, 15 mg / 2ml). They stated that they observed signs of improvement in all 3 groups, did not detect any significant difference between the groups and did not encounter any systemic side effects other than temporary regional side effects. Different to our study, no significant differences were found between the control group and the LMW HA group. In addition to the evaluation criteria, our study is different than this study in terms of follow-up times and application area and the HMWHA we used is 5 Mda in weight and has an adhesive structure. It may be thought that the physical and chemical structure differences of HMWHA used may have affected the difference in the findings.

Unlike our findings, Sato et al. (249) formed a space between the molars of the rats by using separation elastics, and they examined the distribution of versican, which is a type of proteoglycan and HA in the periodontal ligaments compression and tension areas. They reported that after orthodontic force, the distribution of HA showed no difference. HA was unable to penetrate between tissues and this may be because of the HMW structure. In our study, we think that HMWHA preserved its concentrate in the midpalatal suture area because of its adhesive structure and increased the osteoblastic activity. LMWHA might have dispersed in the tissue due to its structure and could not affect bone formation by not maintaining its concentration as good as HMWHA

HA, which is one of the main substances of ECMs of all living tissues, is also frequently used in the fields of dentistry, orthopedics, dermatology, ophthalmology for its anti-inflammatory and antibacterial effects in recent years (137,138). Based on the positive effects of HMW and LMWHA in our study on the midpalatal suture areas of rats after the rapid maxillary expansion and other studies on bone formation, it can be used in order to accelerate bone formation in the midpalatal suture area of humans. It can be considered that it can be applied locally and systemically thereby reducing or eliminating the risk of relapse.

When we compared the average bone volume between the 10 and 20 days experimental groups, we found out that the bone volume in the control group was significantly lower than HMWHA and LMWHA groups. This results coincide with the findings of Arpağ et al. (210), who reported that the HMWHA affects bone healing positively in a short period of time. They found out that high molecular weight hyaluronic acid could contribute to the healing of xenograft by improving the percentage of new bone formation and reducing the percentage of residual graft. However, HA did not significantly affect the quality of newly formed bone assessed by microarchitectural parameters. Sasaki and Watanabe's (176) study also supported this result by reporting that HMWHA increased new bone formation in femoral defects of rats after bone marrow ablation.

When comparing the LMWHA and HMWHA groups among themselves, the bone volume in the LMWHA group was found to be lower. In line with these results, it can be said that the effect of LMWHA substance compared to HMWHA substance is less effective in the formation of new bone. But LMWHA groups shows significantly high results than control groups.

Cyphert et al. (250), reported that when it comes to biological effects of HA, the molecular size matters. LMW and HMW HA can cause different signaling between cells and therefore have different roles in tissue reactions. This might explain the different amount of new bone formation between HMW and LMWHA injections to midpalatal suture area.

Ariyoshi et al. (251) demonstrated that HMWHA has a role in regulating osteoclast formation by down-regulating RANKL expression. They claim that osteoclast quantity can decrease when HMWHA is present in the tissue. In our study, new bone volume in HMWHA

groups were higher than both LMWHA 10 and 20 days groups. This result can be the consequence of lower osteoclast levels in the HMWHA groups.

We think that, in order to access more information about HMW and LMWHA and its effects on midpalatal suture area, we need experiments with long term follow-up times. There are a few studies about usage of HA on midpalatal suture area and about dentistry subjects. New studies are needed to evaluate effect of HMW and LMWHA systemically and regionally in terms of dose to be administered.



6. CONCLUSION

- In all rats applied with helix spring, skeletal expansion was obtained.
- Weight loss occurred in all groups after the 5 day expansion period. Rats in all experimental groups gained weight in retention period.
- Total bone and connective tissue volume increased in HMW HA and LMW HA 10 and 20 day groups according to control groups.
- HMW HA 10 and 20 day groups showed the highest bone and connective tissue values.
- The 10 and 20 day group with LMW HA had higher bone and soft tissue volumes than the control groups, but lower volume than the HMW HA groups.
- There are no significant differences between all groups in the biochemical analysis of the blood serum plasma HA levels.

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8. APPENDICES

8.1. Ethical Approval



T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU (YÜDHEK)

ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
15.06.2015	471	02.06.2015 Tarihli Yazı	Doç.Dr. Didem NALBANTGİL

“Farklı molekül ağırlıklı hyaluronik asidin sıçanlarda kemik oluşumuna olan lokal ve sistemik etkisinin histomorfometrik ve biokimyasal olarak incelenmesi” adlı bilimsel çalışma etik kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oy birliğiyle karar verilmiştir.

Etik Onay Geçerlilik Süresi: 1 Yıl

GÖREVİ	ADI SOYADI	İMZA
Başkan	Prof. Dr. M. Ece GENÇ	
Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA	
Raportör	Prof. Dr. Işıl Aksan KURNAZ	
Üye	Prof. Dr. Bayram YILMAZ	
Üye	Prof. Dr. Başar ATALAY	
Üye	Yard.Doç.Dr.Soner DOĞAN	
Üye	Yard. Doç. Dr. Ediz DENİZ	
Üye	Doç. Dr. C. Narter YEŞİLDAĞLAR	
Üye	Sumru KİRAZCI	

9. CURRICULUM VITAE

Kişisel Bilgiler

Adı	ALİ	Soyadı	GÖRDEAL
Doğum Yeri		Doğum Tarihi	
Uyruğu		TC Kimlik No	
E-mail		Tel	

Öğrenim Durumu

Derece	Alan	Mezun Olduğu Kurumun Adı	Mezuniyet Yılı
Doktora	ORTODONTİ	YEDİTEPE ÜNİVERSİTESİ	2022
Yüksek Lisans	-		
Lisans	DİŞ HEKİMLİĞİ	YEDİTEPE ÜNİVERSİTESİ	2011
Lise	-	PERTEVNİYAL ANADOLU LİSESİ	2006

Başarılmış birden fazla sınav varsa (KPDS, ÜDS, TOEFL; EELTS vs), tüm sonuçlar yazılmalıdır

Bildiği Yabancı Dilleri	Yabancı Dil Sınav Notu (#)
İNGİLİZCE	ÜDS 2011 Bahar(73,75), ÜDS 2010 Sonbahar(70)

İş Deneyimi (Sondan geçmişe doğru sıralayın)

Görevi	1.1.1 Kurum	Süre (Yıl - Yıl)
		-
		-

Bilgisayar Bilgisi

Program	Kullanma becerisi
MICROSOFT OFFICE	İYİ

*Çok iyi, iyi, orta, zayıf olarak değerlendirin

Bilimsel Çalışmaları

SCI, SSCI, AHCI indekslerine giren dergilerde yayınlanan makaleler

-
-

Diğer dergilerde yayınlanan makaleler

-
-

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

Hakemli konferans/sempozyumların bildiri kitaplarında yer alan yayınlar

Diğer (Görev Aldığı Projeler/Sertifika/Ödülleri)
