

Injectable hyaluronic acid: a method for accelerating alveolar bone remodelling during orthodontic tooth movement

Dler Mourad,^{*} Merve Goymen,[†] Fatma Deniz Uzuner,[‡] Tuna Onal[§] and Mehmet Ibrahim Tuglu[‡]

Private Practice, Gaziantep, Turkey^{*}

Department of Orthodontics, Faculty of Dentistry, Gaziantep University, Gaziantep, Turkey[†]

Department of Orthodontics, Faculty of Dentistry, Gazi University, Ankara, Turkey[‡]

Department of Histology and Embryology, Faculty of Medicine, Bandirma Onyedi Eylul University, Balikesir, Turkey[§]

Department of Histology and Embryology, Faculty of Medicine, Manisa Celal Bayar University, Manisa, Turkey[‡]

Objective: The aim of this study was to evaluate the effects of high molecular weight hyaluronic acid (HMWHA) and low molecular weight hyaluronic acid (LMWHA) on alveolar bone remodelling during orthodontic tooth movement (OTM).

Materials and methods: The study involved 60 Wistar-Albino rats, divided into two main groups and further divided based on time periods (1, 3, 7, 14, and 21 days) therefore containing 6 rats in each subgroup. Orthodontic mesialisation of the maxillary first molars was bilaterally achieved using nickel-titanium closed coil springs. In Group I, the right side was injected with HMWHA, while in Group II, the right side was injected with LMWHA. The left side of all rats was injected with normal saline. OTM was measured using 3-dimensional digital models. Alveolar bone volume in the interradicular areas of the first molars was evaluated through histomorphometric and immunohistochemical analyses of receptor activator of nuclear factor- κ B ligand (RANKL) and osteoprotegerin (OPG).

Results: Histologically, on days 14 and 21, the number of osteoclasts on the experimental sides was higher than on the control sides in both groups. On day 21, the amount of OTM in the HMWHA-injected group was 1.4 times greater than in the control group. Both types of hyaluronic acid increased the RANKL/OPG ratio.

Conclusion: The injection of HA may accelerate orthodontic tooth movement by decreasing alveolar bone density and enhancing bone cell proliferation.

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Dler Mourad: dlermourad@gmail.com; Merve Goymen: mervegoymen@gmail.com; Fatma Deniz Uzuner: fduzuner@yahoo.com.tr; Tuna Onal: tunaonal39@gmail.com; Mehmet Ibrahim Tuglu: mituglu@yahoo.com

Introduction

Hyaluronic acid (HA) is a unique compound that, despite its simple chemical structure, possesses distinctive properties. It consists of large, negatively charged polysaccharides that participate in various biological processes, acting as either a passive molecule or as a signalling molecule. The

hydrophilic nature and viscoelasticity of HA are responsible for its passive action by regulating tissue hydration, modulating the osmo-regulation process and maintaining the components of extracellular matrices. In addition, the ability of HA binding to specific proteins on the same cell or neighbouring cells leads to the stimulation of a signalling pathway

for inflammatory events, plus the differentiation and migration of cells. It has been emphasised that the effect mechanisms of HA are related to a great extent on its molecular weight (MW) that plays a crucial role in the mode of action.¹⁻³

As a biocompatible material, HA applications have been investigated within different medical fields, while its effect on bone tissue has been assessed in vitro which has indicated a significant role in the differentiation of osteoclasts.⁴ The rate of cell adhesion is also inversely affected by HA.¹ This remarkable substance has been proven to be effective in accelerating the bone healing process after tooth extraction by enhancing re-vascularisation, osteo-induction, osteo-conduction and remodelling.⁵ HA has demonstrated a strong bone-repair capability in critical size bone defects by inducing almost complete healing within 2 weeks through the promotion of osteoblastic cell adhesion and calcium binding.⁶

The long duration of orthodontic treatment, often lasting between 1.5 and 3 years, is a major concern for both patients and clinicians.⁷ A shorter treatment time is preferred as it reduces the harmful side effects of orthodontic treatment, such as external apical root resorption,⁸ poor oral hygiene related issues like periodontitis, gingivitis,⁹ the development of white spot lesions, and a higher prevalence of caries.¹⁰ Another critical factor for consideration is the financial aspect of extended orthodontic treatment, so accelerated treatment is more cost effective for the orthodontist and the patient.¹¹ Consequently, the acceleration of orthodontic treatment has attracted significant interest among clinicians and researchers. During orthodontic tooth movement, bone formation and bone destruction occur and the rate of this remodelling cycle is the determining factor governing the duration of orthodontic treatment.¹² The bone remodelling process is driven by bone cells (osteoblasts, osteoclasts and osteocytes); while osteoclasts resorb the bone on the compression side, osteoblasts form new bone on the tension side.¹³

In the light of previous in vitro and clinical studies regarding the correlation of HA with bone tissue, the present study was conducted based on a hypothesis that HA might influence the rate of OTM by regulating alveolar bone remodelling. A review of the literature revealed a limited number of studies that evaluated the relationship between tooth movement and hyaluronic acid. A past study

employed histochemical methods to analyse the distribution of HA within the periodontal ligament during orthodontic tooth movement in rats.¹⁴ The research design analysed the molecules expressed during experimental orthodontic tooth movement to define the histochemical changes in the natural process rather than examine accelerated tooth movement. A related study failed to assess the effect of HA molecules of different weights.¹⁵ Additionally, the conducted histological evaluation did not include an expression analysis of key molecules associated with tooth movement, and was limited to a basic observational assessment.¹⁵

In light of the paucity of the existing literature, the present study, aimed to determine the effect of HA on tooth movement by identifying the optimal molecular weight HA through histomorphometric and immunohistochemical analysis, with the expectation of providing a significant contribution to this field of research.

Materials and methods

The experimental protocol was reviewed and approved by the Committee of Experiment Animal Care in Gaziantep University. (Protocol no: 2019/3/82). Sixty male Wistar-Albino rats (277.35 ± 50.16 g, 10 weeks old) were obtained and accommodated in the University Laboratory Animal Science Centre. The rats were kept in polycarbonate boxes at temperatures between 19°C and 22°C, and humidity of 40% to 50%, in a photoperiod of 12/12 hr starting at 8.00 am.

The rats were randomly allocated into two groups in a split mouth study with the right side designated as the experimental side and the left side as a control. Each group was divided into five subgroups of six rats according to the schedule of 1, 3, 7, 14, and 21 days. An orthodontic model was established by the bilateral mesialisation of the maxillary molars as previously described.¹⁶ All the procedures were performed under general anesthesia using a combination of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (100 mg/kg). An impression of the maxilla was taken using vinyl polysiloxane material (Oran wash L, Zhemarck, Rovigo, Italy) in a custom-made impression tray which was kept at room temperature for not more than 3 hr prior to scanning and digital modelling.

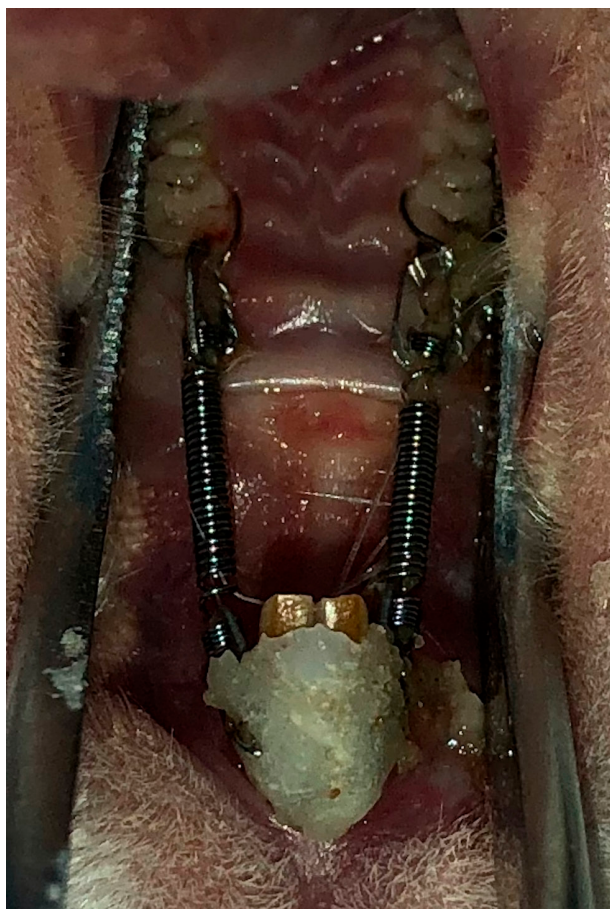


Figure 1. Orthodontic appliance in situ.

Nickel titanium closed coil springs (G&H Orthodontics, Franklin, USA) were applied to provide an orthodontic force. The closed coil spring was fixed to the incisors with a ligature wire (0.08 inch) passed through a hole of 0.5 mm diameter drilled perpendicular to the long axis at the gingival level of incisors as shown in Figure 1. An applied force of 40g was measured by a dynamometer (Correx, Haag-Streit, Berne, Switzerland) as described in previous literature.¹⁷ In order to stabilise the appliance and prevent soft tissue damage, light cured glass ionomer cement (3M Unitek™ Multi-Cure Glass Ionomer Orthodontic Band Cement, Monrovia, California, USA) was placed on the posterior and anterior end of the ligature wire with no further activation of the appliance after the initial delivery. The lower incisors were trimmed by a metal diamond disk (G&H Orthodontics, Franklin, USA) using a micro drill (Omnidril 35, World Precision Instruments,

Sarasota, Florida, USA) to prevent breakage of the appliance. Because of continuous eruption of the lower incisors in rodents, the trimming procedure was repeated every three days and the appliance was checked daily for breakage. After placement of the closed coil springs, HMWHA (Synvisc, Sanofi-Aventis, Quebec, Canada) (6000kDa) was injected into the right side of Group I; LMWHA (Hyalrat, Fidia Farmaceutici S.P.A, Padua, Italy) (500 to 750 kDa) was injected into the right side of Group II, while 0.9% NaCl was injected to the left side of both groups. The HA agent and 0.9% NaCl were injected into the buccal sulcus adjacent to the upper 1st molar at a dose of 10 µl which is the maximum amount accepted by the parodontal tissues without overflow.

During the experimental period, the general condition of the rats and their orthodontic appliances were checked daily and the animals were fed pellets in powder form and water, ad libitum. All the animals were weighed daily which revealed a decrease in their body weight for 3 days after placement of the appliance before recovery. The rats with declining general condition or with a broken appliance were replaced by rats of the same weight and age and a repetition of the experimental steps before exclusion.

Immediately after the removal of the traction appliance from the maxilla, the animal was sacrificed by a high dose of anesthetic solution and an impression was retaken and digitally scanned on the same day. Digital scanning and OTM measurements were performed using 3Shape Lab Scanner (D-250; 3Shape, Copenhagen, Denmark) and 3Shape Dental System Premium Software Version 19.1 (3Shape, Copenhagen, Denmark). After scanning, the 3D images were transferred to Dental CAD software (Version 2016.10 Valetta, Exocad, Darmstadt, Germany). Tooth movement measurements were conducted on the digital models obtained from the impressions taken before and after the procedure. For the superimposition, the peaks of the first, second, and third maxillary rugae on both the right and left sides were identified as reference points. The points were consistent and unaffected by tooth movement nor orthodontic treatment, making them suitable for precise superimposition. This is visually represented in Figure 2, in which the blue points indicate the reference points for the superimpositions. All the measurements were conducted along the occlusal plane.

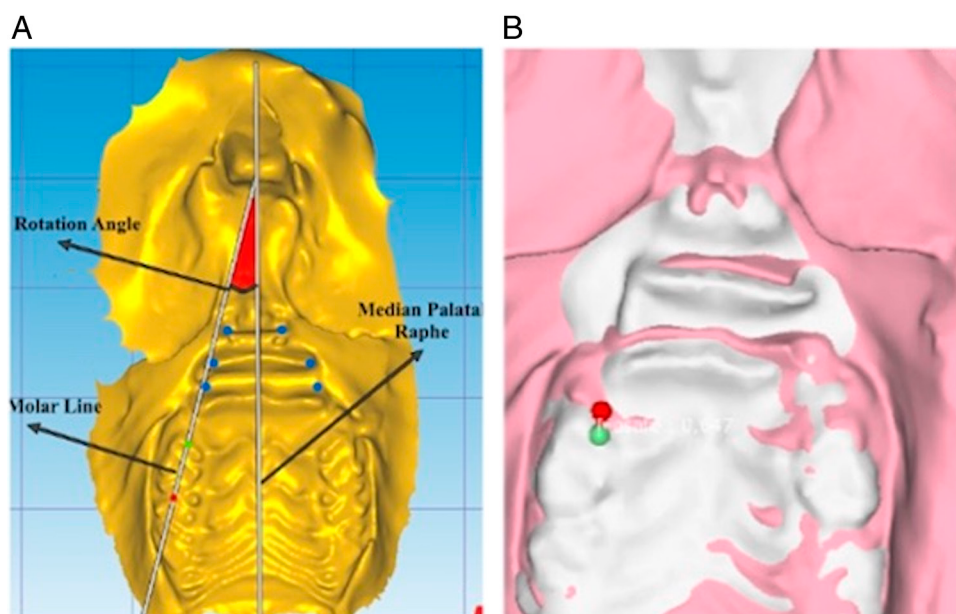


Figure 2. 3D model: A, The blue points refer to the superimposition points and measurements of molar rotation around its long axis. B, Measurement of orthodontic tooth movement, green point refer to T0, red point refer to T1.

Additionally, the first molar's mesial marginal ridge midpoint was marked separately on the pretreatment and posttreatment models, which allowed the measurement of tooth movement between these two points, as described previously.¹⁸ During the mesialisation of the maxillary 1st molar by the closed coil spring, axial rotation of the tooth may affect the accuracy of the tooth movement measurements. To account for this, the angle formed between two lines: one passing through the distal and mesial contact points of the 1st molar and the other passing through the palatal suture, was measured. The angle was determined on the digital impressions at T0 and T1, and the molar rotation was calculated as previously described.¹⁹

The dissected maxilla was separated into two halves along the mid-palatal suture by a surgical blade. Tissue samples were fixed in 10% neutral formaldehyde for 2 to 3 days, following which samples were thoroughly washed with phosphate-buffered saline (PBS) to remove formalin and decalcified by a hydrochloric acid/formic acid solution (Biodec R, Bio-Optica, Milano, Italy), which was changed once a week for 3 weeks.

The decalcified tissues were dehydrated through a series of alcohol concentrations before being histologically prepared and embedded in paraffin blocks. Each block

was cut longitudinally parallel to the long axis of the first molar at a thickness of 5 μm up to the level of the radicular pulp. Three of the prepared histological slides in each sample, which were taken from the beginning, middle, and end of the sections, were stained with haematoxylin and eosin (HE).

Stained specimens were viewed under a light microscope (Nikon Eclipse E400, Nikon, Tokyo, Japan). For each specimen, the same area was captured, and transferred to a computer and calibrated with a Nikon micro-meter slide (Nikon, Tokyo, Japan). All photographs were evaluated using Clemex Vision Lite Image Analysis 3.5) (Clemex Technologies, Longueuil, Canada). An area of 1.5 μm^2 in the interradicular area of each section was examined in both the experimental and control group.

The osteoblastic cells were considered as those which had a cuboid shape with basophilic cytoplasm and a polarised nucleus, while the osteoclasts were identified by noting cells sited near bone and which had an irregular outline and multiple nuclei.

The number of osteoclasts were scored in three areas for every stained slide and included the distal aspect of the mesial root, the mesial aspect of the distal root and the furcation area. The parameters were scored as +1 (none or less than 3 cells), +2 (3 to 5 cells), +3 (6 to 8 cells), +4 (8 to 10 cells) and +5 (more than 10 cells)

by two researchers who were blinded to the nature of the samples.

The volume of the alveolar bone was stereologically measured using the Image J program (Bone J2 plugin, version 1.5, Wyne Rasband). The surface area of the alveolar bone in each section was calculated and multiplied by the thickness of the 5 μm section to determine the bone volume of each section. Total bone volume of the sample was estimated by multiplying the calculated volumes by the section sampling fraction. To assess bone density, the bone volume to total volume ratio (BV/TV) was calculated. The ratio represents the proportion of the bone volume relative to the total volume of all structures including alveolar bone, periodontal ligament, connective tissue and bone marrow cavities as previously described.¹⁷

For a more detailed assessment of osteoclastic activity, an immunohistochemical analysis of RANKL and OPG was performed. The specimen was cleared with xylene (30 min), rehydrated in alcohol, and placed in distilled water (5 min). Sections were treated with 0.5% trypsin (15 min), blocked with 3% hydrogen peroxide (5 min), and washed three times in phosphate buffer saline (PBS). After immersing in blocking serum (1 h), the primary antibodies were incubated overnight. The next day, the sections were re-washed in PBS, stained with streptavidin-biotin secondary antibody (Zymed Histostain kit San Francisco, USA) for 30 min, and treated with DAB (5 min). Finally, sections were counterstained with Mayer's haematoxylin (72804E, Microm, Walldorf, Germany), washed in distilled water, and mounted in permanent media.

Catabolic and anabolic activities of alveolar bone were assessed by evaluating the osteoclastogenic expression of RANKL and OPG which is a decoy receptor that inhibits osteoclast activity and favours bone preservation. The intensity and distribution of RANKL and OPG staining were quantified using an H-score, which considers both the intensity and the percentage of cells expressing each marker. This score provides a numeric measure of RANKL and OPG levels and serves as a critical indicator of bone remodelling status, in which a higher ratio indicates increased resorptive activity, and a lower ratio suggests anabolic, bone-preserving conditions. The stained cells were counted as (0) = negative; (1) = weak positive; (2) = positive; (3) = strong

positive, and evaluated with an H-score that was obtained by the formula:²⁰

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

A statistical analysis was conducted using SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA). In each group, the descriptive mean and standard deviation of each parameter were determined. The compatibility of numerical variables to normal distribution was tested by applying the Shapiro–Wilk test. The paired Student's *t* test was used in a comparison of normally distributed variables in two dependent measurements. An unpaired Student's *t* test was used in a comparison of normally distributed variables in two independent groups. A significance level of $p < 0.05$ was applied for all statistical comparisons and the results were evaluated at a confidence interval of 95%.

Results

The reliability of the measurements was determined by the intraclass correlation coefficient values of bone density and OTM which were above 0.9 for all subgroups. In the HMWHA injected sides, a statistically significant increase in tooth movement from the 1st to the 21st day occurred, while LMWHA showed a statistically significant increase in tooth movement from the 3rd to the 21st day when compared to the control side ($p < 0.05$) (Table I). Even though the level of OTM in the HMWHA injected side was greater than the LMWHA injected side, no significant difference was found between the two until day 21 when HMWHA revealed greater significant tooth movement than LMWHA ($p < 0.001$) as shown in Table II and illustrated in Figure 3.

As a result of the histomorphometric analysis of the alveolar bone, no significant difference was found between the experimental and control sides until day 14 at which time a significant decrease in the BV/TV ratio was found ($p < 0.05$). The bone formation increased again between day 14 and day 21 in all groups but was still significantly lower than the control side ($p < 0.05$) (Table III). No significant differences were noted between the high and low MW groups following a comparison of the intergroup results for the BV/TV ratio.

The number of osteoclasts increased significantly in the HA injected tissues in comparison to the control

Table I. Comparison of tooth movements between experimental and control sides on each observational day (Mean $\pm$ SD) (mm)

Group	Experimental	Control	<i>P</i>
Group I- Day 1	0.046 $\pm$ 0.009	0.026 $\pm$ 0.01	0.002*
Group I- Day 3	0.469 $\pm$ 0.024	0.341 $\pm$ 0.028	0.001**
Group I- Day 7	0.67 $\pm$ 0.024	0.456 $\pm$ 0.028	0.001**
Group I- Day 14	0.989 $\pm$ 0.045	0.54 $\pm$ 0.047	0.001**
Group I- Day 21	1.143 $\pm$ 0.054	0.93 $\pm$ 0.048	0.001**
Group II- Day 1	0.033 $\pm$ 0.008	0.026 $\pm$ 0.007	0.06
Group II- Day 3	0.45 $\pm$ 0.039	0.346 $\pm$ 0.026	0.001**
Group II- Day 7	0.637 $\pm$ 0.039	0.444 $\pm$ 0.026	0.001**
Group II- Day 14	0.964 $\pm$ 0.067	0.552 $\pm$ 0.056	0.001**
Group II- Day 21	0.977 $\pm$ 0.035	0.866 $\pm$ 0.037	0.001**

Paired Student's *t* test, Significance level **p* $\leq$ 0.01, ***p* $\leq$ 0.001.

Table II. Intragroup Comparison of OTM Amount (Mean $\pm$ SD) (mm)

	Group	Day 1	Day 3	Day 7	Day 14	Day 21
Experimental Side	HMWHA	0.046 $\pm$ 0.009	0.47 $\pm$ 0.024	0.67 $\pm$ 0.024	0.989 $\pm$ 0.045	1.143 $\pm$ 0.054
	LMWHA	0.033 $\pm$ 0.008	0.45 $\pm$ 0.039	0.637 $\pm$ 0.039	0.964 $\pm$ 0.067	0.977 $\pm$ 0.035
	<i>p</i>	0.009*	0.165	0.07	0.233	<0.001**
Control Side	HMWHA	0.026 $\pm$ 0.01	0.341 $\pm$ 0.028	0.456 $\pm$ 0.028	0.54 $\pm$ 0.047	0.93 $\pm$ 0.048
	LMWHA	0.026 $\pm$ 0.007	0.346 $\pm$ 0.026	0.444 $\pm$ 0.026	0.552 $\pm$ 0.056	0.866 $\pm$ 0.037
	<i>p</i>	0.493	0.374	0.133	0.347	0.07

Unpaired Student's *t* test, Significance level **p* $\leq$ 0.01, ***p* $\leq$ 0.001.

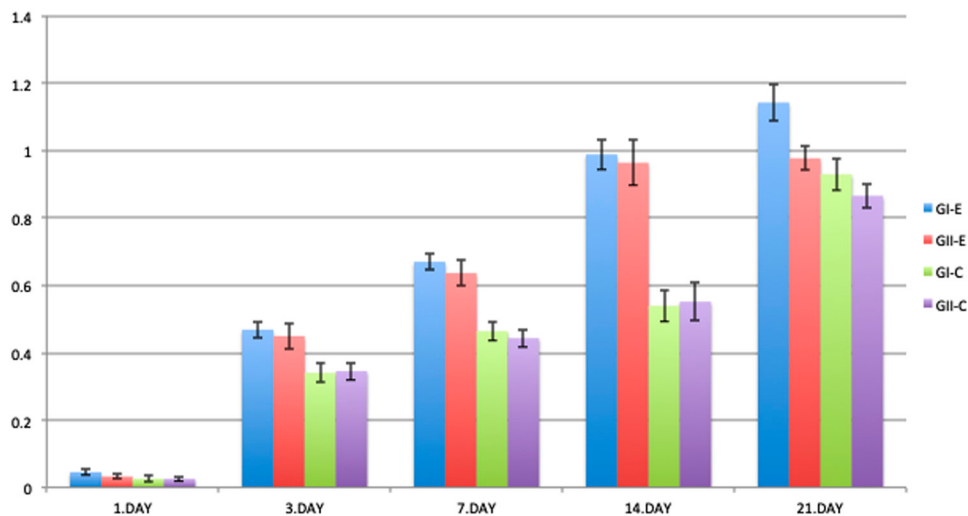
**Figure 3.** Comparison of OTM amount in all time points between groups I and II (E): experimental, (C) control.

Table III. BV/TV Percentage in Experimental and Control Groups (Mean $\pm$ SD) (%)

Group	Experimental	Control	<i>p</i>
Group I- Day 1	47.254 $\pm$ 2.528	47.270 $\pm$ 2.205	NS
Group I- Day 3	43.458 $\pm$ 2.44	42.050 $\pm$ 1.522	NS
Group I- Day 7	29.164 $\pm$ 3.846	31.296 $\pm$ 3.828	NS
Group I- Day 14	25.594 $\pm$ 3.562	31.088 $\pm$ 3.222	0.05*
Group I- Day 21	32.111 $\pm$ 0.7840	39.890 $\pm$ 2.068	0.05*
Group II- Day 1	47.884 $\pm$ 2.340	45.846 $\pm$ 2.727	NS
Group II- Day 3	46.089 $\pm$ 3.447	46.168 $\pm$ 3.042	NS
Group II- Day 7	26.818 $\pm$ 3.910	27.026 $\pm$ 3.489	NS
Group II- Day 14	26.379 $\pm$ 3.757	32.386 $\pm$ 3.825	0.05*
Group II- Day 21	36.071 $\pm$ 2.632	39.459 $\pm$ 2.028	0.05*

Paired Student's *t* test, Significance level **p* $\leq$ 0.05, NS; nonsignificant.

sides on days 14 and 21 which matches the bone volume results (Table IV). Representative images of histological sections are shown in Figure 4.

The RANKL/OPG ratio in the HA experimental side was found to be significantly higher than the control sides at days 14 and 21 (*p* $<$ 0.05) (Table V). A gradual increase in the RANKL/OPG ratio was observed from baseline towards day 21 that showed higher significance on day 7 in LMWHA group and on day 21 in HMWHA group as illustrated in Figure 5. Representative histologic images of RANKL and OPG expression are shown in Figure 6.

Discussion

An acceleration of OTM can be achieved by increasing the number of the involved bone cells in alveolar bone remodelling to accelerate the resorption/formation cycle or by decreasing the bone resistance to tooth movement. HA is an agent involved in bone remodelling and has an important role in osteoclast proliferation.⁴ According to past research, the MW of HA plays an important role in its efficacy.^{1,2} The present study was therefore conducted to evaluate and compare the effect of the local injection of high and low MW HA on alveolar bone density through the modification of bone cytokine mediators and the resultant effect on the rate of OTM. To assess bone response in vivo, the acceleration effect of HA on tooth movement was revealed in a rat model.

Table IV. Number of Osteoclasts in Study Groups (Mean $\pm$ SD) (+1/+5 Score)

	Experimental	Control	<i>p</i>
Group I- Day 1	1.35 $\pm$ 0.17	1.34 $\pm$ 0.19	<i>p</i> $>$ 0.05
Group I- Day 3	2.01 $\pm$ 0.32	1.89 $\pm$ 0.27	<i>p</i> $>$ 0.05
Group I- Day 7	3.83 $\pm$ 0.41	2.5 $\pm$ 0.55	<i>p</i> $>$ 0.05
Group I- Day 14	5 $\pm$ 0.89	2.5 $\pm$ 0.57	<i>p</i> $<$ 0.001**
Group I- Day 21	6.33 $\pm$ 0.81	3 $\pm$ 0.89	<i>p</i> $<$ 0.001**
Group II- Day 1	1.41 $\pm$ 0.23	1.36 $\pm$ 0.21	<i>p</i> $>$ 0.05
Group II- Day 3	2.13 $\pm$ 0.29	1.74 $\pm$ 0.27	<i>p</i> $>$ 0.05
Group II- Day 7	3.17 $\pm$ 0.75	2.33 $\pm$ 0.52	<i>p</i> $>$ 0.05
Group II- Day 14	4.67 $\pm$ 1.21	2.83 $\pm$ 0.75	<i>p</i> $<$ 0.05*
Group II- Day 21	5.17 $\pm$ 1.17	3.33 $\pm$ 0.81	<i>p</i> $<$ 0.05*

Paired Student's *t* test, Significance level **p* $<$ 0.05, **Significance level *p* $\leq$ 0.001.

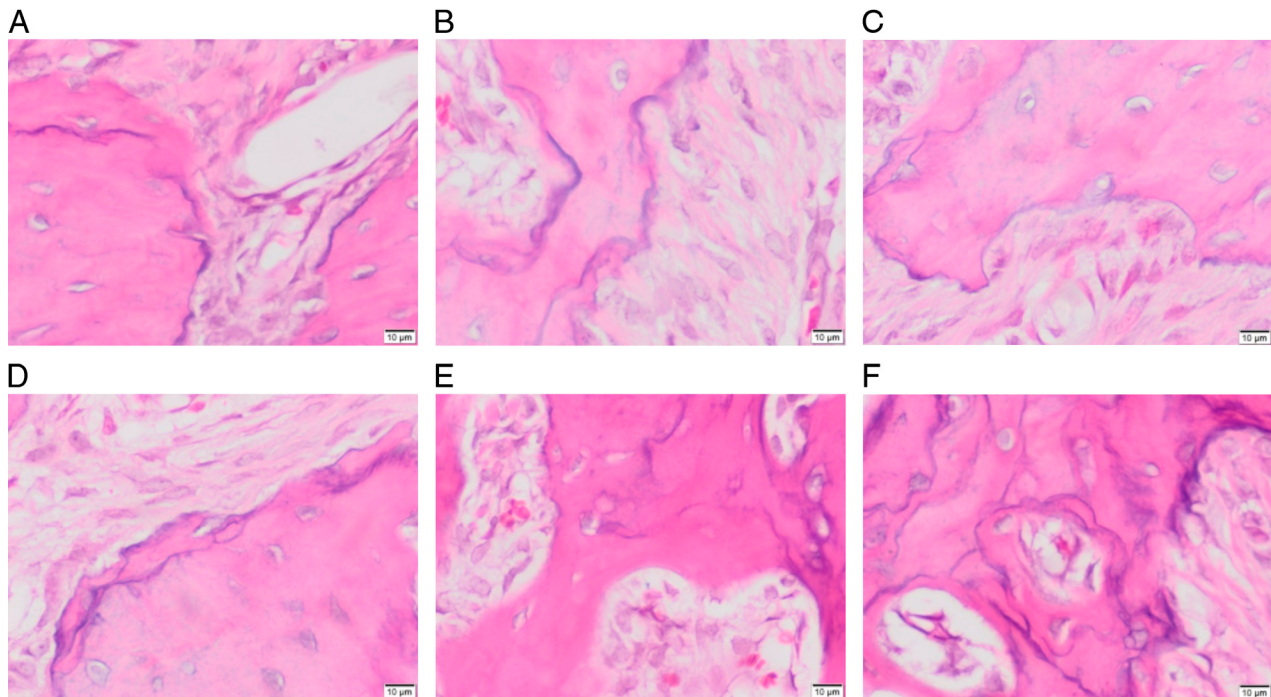


Figure 4. Histologic images of osteoclast cells in interradicular area of maxillary molar on day 14 and 21: A, GI experimental side on day 14. B, GII experimental side on day 14. C, Saline injected side on day 14. D, GI experimental side on day 21. E, GII experimental side on day 21. F, Saline injected side on day 21.

Two main techniques were used for calculating the bony parameters in previous studies: the stereological method and the microtomography method^{17,18,21}. Gulec et al. calculated the BV/TV ratio in the interradicular area of the 1st molar by stereological analysis to assess alveolar bone density.¹⁶ Meh et al. estimated bone volume density around the five roots of the rat 1st molar by a point counting stereological method.¹⁷ Additional studies used micro-computed tomography to measure the bone parameters of volume and density.^{18,21} In the present study, the stereological analysis of bone tissue was used for the interpretation of three-dimensional bone parameters from two-dimensional sections due to limitations of micro-tomography related to artefacts associated with radiation,²² the difficulty in achieving a standard position for the scanning of the animals,²³ and the effect of radiation dose on bone metabolism that described a loss of about 30% of BV/TV ratio in mice.²⁴

The results of the present study demonstrated that the changes in BV/TV began on day 7 and were more significant after day 14 at the later phase of orthodontic tooth movement. In the control

groups, decreases in BV/TV also started as early as day 7, but a lag phase was seen between days 7 and 14. A substantial number of published studies have documented a notable decline on the seventh day, a finding that is consistent with the results of the present study.^{21,25} The observed reduction in bone density can be interpreted as aiding the acceleration of tooth movement, which occurs in parallel with the reduction in the BV/TV ratio likely associated with the elevated osteoclastic activity. Accordingly, in the control groups, the highest osteoclast counts were seen on day 21. Both catabolic and anabolic activities of bone are the determining factors governing OTM, but the amount of bone resorption in the direction of movement is the key factor in the acceleration of tooth movement.²⁶ The OTM begins as 1st phase tooth movement inside the socket followed by bone resorption and apposition during the 2nd phase. Frost et al. determined that the bone response to external stimuli leads to an increase in the rate of local remodelling to repair bone in a process known as a regional accelerative phenomenon (RAP).²⁷ The histologic finding of early and rapid bone resorption in the HMWHA and LMWHA groups led to the

Table V. RANKL/OPG Ratio in Study Group (Mean $\pm$ SD)

	Experimental	Control	<i>p</i>
Group I- Day 1	1.08 $\pm$ 0.09	1.14 $\pm$ 0.23	0.336
Group I- Day 3	1.14 $\pm$ 0.16	1.1 $\pm$ 0.23	0.387
Group I- Day 7	1.06 $\pm$ 0.25	1.01 $\pm$ 0.23	0.297
Group I- Day 14	1.17 $\pm$ 0.16	1.14 $\pm$ 0.18	0.044*
Group I- Day 21	1.45 $\pm$ 0.16	1.21 $\pm$ 0.15	0.03*
Group II- Day 1	1.01 $\pm$ 0.15	1.01 $\pm$ 0.15	0.481
Group II- Day 3	1.21 $\pm$ 0.27	1.05 $\pm$ 0.16	0.161
Group II- Day 7	1.2 $\pm$ 0.18	0.91 $\pm$ 0.12	0.021*
Group II- Day 14	1.28 $\pm$ 0.1	1.11 $\pm$ 0.14	0.043*
Group II- Day 21	1.37 $\pm$ 0.12	1.25 $\pm$ 0.18	0.048*

Paired Student's *t* test, Significance level **p* < 0.05.

hypothesis that HA administration created a RAP-like effect which forms the basis of rapid tooth movement. This finding was noted as more rapid OTM in the HMWHA and LMWHA injected groups than in the control sides on all observational days. At the end of day 21, the injected HMWHA group showed 1.4 times greater OTM than the control side and 1.2 times greater OTM than the LMWHA side.

The HA of both high and low MW increased the OTM significantly. HMWHA accelerated the OTM in a rate higher than LMWHA on day 21, which may be attributed to the greater enzymatic events required for HMWHA to be cleaved to smaller size oligomer, and therefore extend the effect of HA on bone turnover.²⁸ A previous study by Sadikoglu et al. found that HMWHA increased bone formation in the intermaxillary suture while LMWHA had no effect.² These results were contrary to the outcomes of the present study, which could be explained by

the different region of force application. Although osteoclast numbers in the injected HA sides were higher than the control sides on day 14 and 21, the recorded tooth movement levels were greater in the experimental HMWHA and LMWHA groups at all experimental days. The findings could be attributed to the bypassing of the lag phase after the displacement phase that is characteristic of tooth movement of conventional orthodontics in the injection groups and could be accepted as a clue regarding the development of a multi-HA injection protocol for more rapid orthodontic tooth movement.

The two different MW of HA that were examined in the present study were determined according to the literature which claimed that HA has a MW dependent effect on bone turnover. Previously, it had been determined that different MW of HA had contrasting effects on osteoclasts, so that LMWHA enhanced osteoclast formation, while HMWHA had no effect on osteoclast differentiation.⁴ The data strongly linked the induction of HA synthesis to osteoclastic resorption, prompting the suggestion that an increased level of HA in the extracellular matrix could enhance bone resorption thereby facilitating accelerated orthodontic treatment. Various MW of HA compounds of which two basic types have been used either HMWHA that have a MW more than 1000 kDa and LMWHA that have a MW less than 1000 kDa.³

The cellular response to different MW of HA had been evaluated by several in vitro, in vivo and clinical studies, which determined that the HA had a

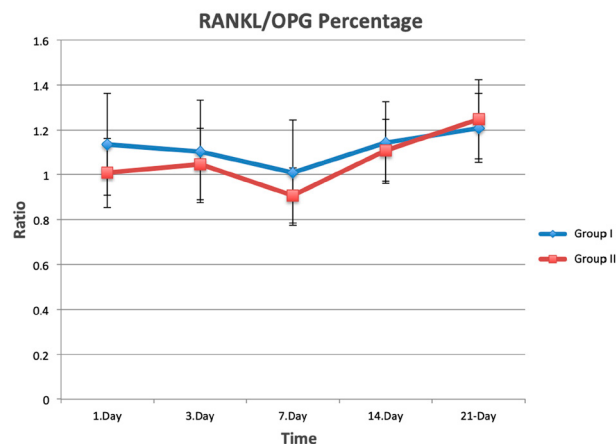


Figure 5. RANKL/OPG ratios at experimental sides of groups I and II in each observation days.

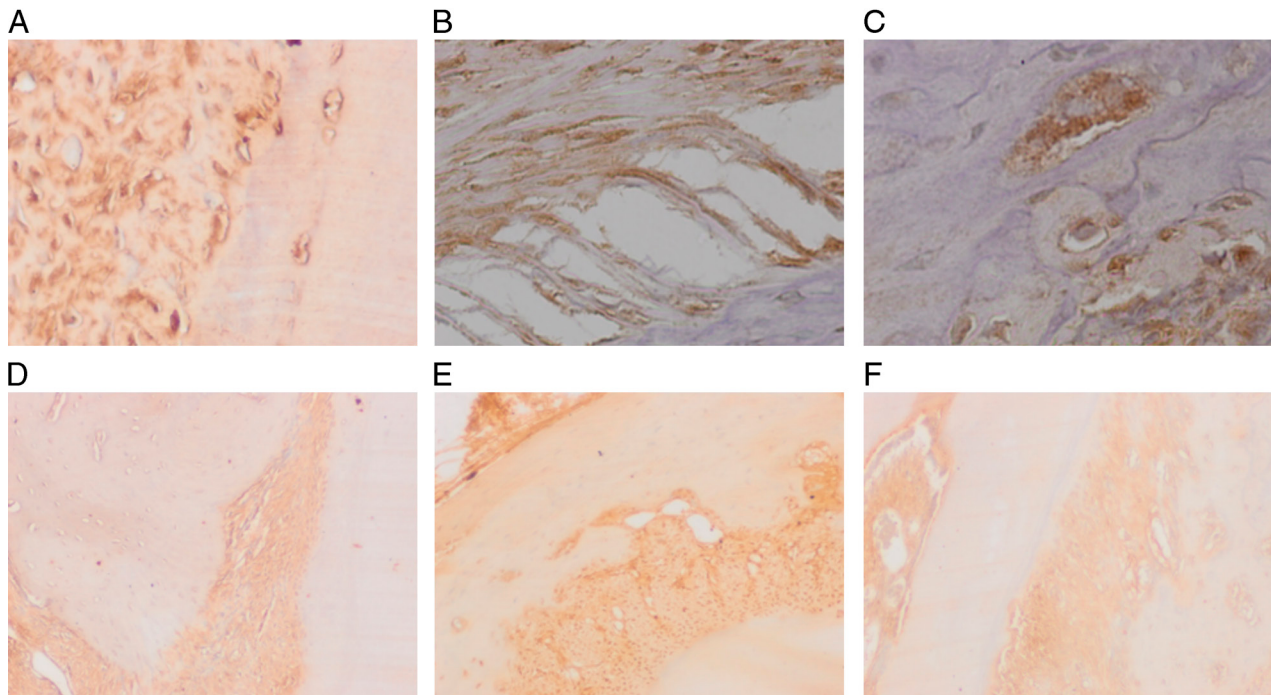


Figure 6. Histologic images of RANKL and OPG expression in interradicular area of maxillary molar on day 21: A, RANKL in GI experimental side. B, RANKL in GII experimental side. C, RANKL in Saline injected side. D, OPG in GI experimental side. E, OPG in GII experimental side. F, OPG in Saline injected side.

MW-specific mode of action on bone formation with 900 to 2300 kDa enhanced bone formation better than 60 kDa HA.²⁹ Related to maxillary expansion, the HMWHA (5000 kDa) increased the rate of bone formation in an expanded intermaxillary suture, while LMWHA (645 kDa) had no effect.² In the present study, the effect of HMWHA with 6000 kDa MW and LMWHA with 500 to 750 kDa MW on bone resorption after OTM, was investigated. To understand the effect of HA on bone remodelling during OTM, a histological examination was performed and an estimate of bone volume density was used to assess the amount of resorbed bone.^{17,18,21}

Cao et al. observed that an addition of exogenous HA to bone marrow stem cell culture improved the expression of RANKL expressed in bone that, in turn, reflected greater osteoclastic activity.³⁰ Ariyoshi et al. found that different molecular weights of HA had contrasting effects on osteoclasts, so that LMWHA enhanced osteoclast formation, while HMWHA had no effect on osteoclast differentiation.³¹ Fujii et al. concluded that the interactions of HA with CD44 protein found on osteoblasts and osteoclasts might play an essential role in bone metabolism, including osteoclastogenesis.³²

Related to bone volume density results, a decrease in BV/TV ratio means an increase in osteoclastic activity and a decrease in the resistance of the bone to the OTM.¹² In the present study, bone volume density decreased significantly after experiment day 14 in both groups injected with HA which may induce an acceleration of orthodontic treatment. The bone density related outcomes lead to the possibility of an increase in osteoclast number in the compression side leading to a decrease in bone volume density in the interradicular area of the involved molar. The calculation of the number of osteoclasts has been used in most studies examining the acceleration of OTM.^{16,33} According to the present findings, osteoclast numbers at the experimental site increased significantly on days 14 and 21 in comparison to the control side, which corresponds to the days on which bone density decreased.

Because of the proved relation between RANKL and OPG in the bone remodelling process, the level of expression of these proteins by immunohistochemical analysis has been evaluated by many studies of OTM.^{33,34} The ratio of the expression of RANKL in bone tissue to that of OPG was a better indicator of the bone remodelling process. In the current study,

the RANKL/OPG ratio was significantly higher in the experimental side than in the control side on days 14 and 21, which means that the bone remodelling balance inclined toward osteoclastogenesis rather than bone formation.

It was observed that the application of an orthodontic force combined with HA injection could act as a stimulus for the RAP and increases the rate of tooth movement.

A review of the literature identified a number of studies that had investigated the impact of local administration of agents, including medications, chemical compounds or platelet-rich plasma, on the relapse of orthodontic treatment.^{2,35,36} Of these, Mohammed et al. reported that magnesium oxide supplementation clinically decreased orthodontic relapse in a rabbit model.³⁵ Sadikoglu et al. described the effect of HA on bone formation after rapid maxillary expansion and concluded that the local injection of high molecular weight HA stimulated bone formation and reduced the risk of relapse after maxillary expansion in rats.² The present study design is constrained by an observation period of 21 days, as its objective was to examine the impact of HA on tooth movement velocity. To gain a more comprehensive understanding of the long-term effect of HA on relapse, further studies with an extended observation period are indicated. Considering that HMWHA accelerated OTM at a rate higher than LMWHA on day 21, it will be beneficial to assess and compare the OTM rate at a time period of more than 21 days. Further studies are therefore needed to assess whether time may be a factor in the differential response to HA of various MW.

Conclusion

- High (6000 kDa) and low (500 to 750 kDa.) molecular weight HA injections resulted in an increase in the rate of OTM throughout all the experimental days.
- The amount of tooth movement in the experimental side of the HMWHA group was 1.4 times greater than the control side and 1.2 times greater than the experimental side of LMWHA group.
- Both high and low molecular weight HA injections resulted in a decrease in interradicular bone density.
- The RANKL/OPG pathway shows more osteoclastogenesis in groups injected with HA than with RANKL thereby increasing at a rate greater than OPG.
- Hyaluronic acid can be considered as a viable stimulus for the acceleration of orthodontic tooth movement.

Conflict of interest

The authors declare that there is no conflict of interest.

Corresponding author

Dr. Merve Goymen, Assoc Prof, Department of Orthodontics, Gaziantep Üniversitesi, Diş hekimliği Fakültesi, Osmangazi, Üniversite Blv., 27310 Şehitkamil/Gaziantep, FAX: +90 342 361 03 46, Telephone: +903423609600. Email: mervegoymen@gmail.com

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