

## OPG-RANKL Levels After Continuous Orthodontic Force

### Devamlı Ortodontik Kuvvet Sonrası OPG-RANKL Seviyeleri

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#### ABSTRACT

**Objective:** To evaluate the levels of osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-β ligand (RANKL) in tension (mesial) and pressure (distal) sides during continuous orthodontic force.

**Methods:** Eighteen canine teeth of 9 adults (5 female, 4 male; age range 17.5-18.9 yrs) with different Angle classifications were included. After first premolar extractions, the maxillary canines were tipped distally by sentalloy closed coil springs with a continuous force of 200 g. Gingival crevicular fluid (GCF) was sampled from mesial and distal gingival crevices of each canine separately at baseline, 1 hour(h), 24 h, 168 h, and 1 month after force application. OPG and RANKL levels were analyzed by ELISA.

**Results:** Concentration of OPG showed an increase in mesial side, while there was a decrease in distal side at 1 h, demonstrating a statistically significant difference ( $p<0.05$ ). RANKL expression declared no significant changes during the study.

**Conclusion:** Local host response towards continuous orthodontic force might lead changes in OPG expression between mesial and distal sides, revealing an acute cellular response of periodontal status depended upon the type of stress.

**Key Words:** Osteoprotegerin, RANKL, orthodontic force, gingival crevicular fluid, distalization, sentalloy coil spring

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#### ÖZET

**Amaç:** Devamlı ortodontik kuvvet sonrasında hareket ettirilen dişlerin gerilim (mezial) ve basınç (distal) bölgelerinde osteoprotegerin (OPG) ve reseptör aktivatör nükleer faktör kappa-β ligand (RANKL) seviyelerinin değerlendirilmesidir.

**Yöntemler:** Farklı molar ilişkiye sahip, çekimli ortodontik tedavi gereksinimi bulunan ve ortalama yaşları 17.5-18.9 yıl olan dokuz bireye ait (5 kız, 4 erkek) toplam 18 kanin dişi araştırmaya dahil edilmiştir. Birinci küçük azı dişlerinin çekimini takiben üst kanin dişleri braketlenmiş ve 200 gr devamlı kuvvet uygulayan sentaloy kapalı yaylar ile distalizasyon kuvveti uygulanmıştır. Diş eti oluğu sıvısı örnekleri, kanin dişlerin mezial ve distal yüzeylerinden kuvvet öncesi, kuvvetten 1 saat, 24 saat, 168 saat ve 1 ay sonra toplanmıştır. OPG ve RANKL seviyeleri ELISA testi ile analiz edilmiştir.

**Bulgular:** OPG konsantrasyonu kuvvet uygulandıktan 1 saat sonra mezial yüzeyde artarken, distal yüzeyde azalmış olup, bu bulgu istatistiksel olarak anlamlı bulunmuştur ( $p<0.05$ ). RANKL seviyeleri çalışma boyunca istatistiksel olarak anlamlı değişim göstermemiştir.

**Sonuç:** Devamlı ortodontik kuvvetler, dişlerin mezial ve distal yüzeylerinde OPG seviyelerinde farklılık göstermekte olup, bu farklılık periodontal dokulardaki akut hücresel cevabın göstergesi olabilir.

**Anahtar Sözcükler:** Osteoprotegerin, RANKL, ortodontik kuvvet, diş eti oluğu sıvısı, distalizasyon, sentaloy kapalı yay

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## INTRODUCTION

During fixed orthodontic treatment, periodontal ligament (PDL) tissues respond rapidly to mechanical stress with consequent metabolic changes which allow tooth movement. These responses are mediated by several inflammatory cytokines and enzymes, responsible for connective tissue remodeling (1, 2). These substances can be monitored by analysis of gingival crevicular fluid (GCF) composition which provides a non-invasive prediction of inflammatory mediators (3-5).

The bone remodeling after orthodontic forces initiates by the activation of vascular changes, synthesis of cytokines, prostaglandins and growth factors. The initial tooth movement is facilitated by an acute aseptic inflammatory reaction. Bone is resorbed in pressure areas on the side toward which the force is directed, and deposited at the tensile areas in the contralateral position. This way, the movement of teeth through bone is facilitated (1-6). Investigations in bone biology identified cytokines/cytokine receptors within the tumor necrosis factor (TNF) family that are required for the control of bone remodeling (4, 5, 7-10). Osteoclastogenesis is primarily induced by one of these receptors, receptor activator of nuclear factor kappa-B ligand (RANKL), which binds to receptor activator of nuclear factor kappa-B (RANK), that is located on the surface of osteoclasts and osteoclast precursors. This binding mediates the attachment of osteoclasts to bone and promotes their survival (11). The activity of RANKL is controlled by a soluble decoy receptor, osteoprotegerin (OPG), which inhibits RANKL activation of osteoclastogenesis (11-13).

Recent studies have focused on the role of RANKL and OPG in periodontal diseases and during orthodontic tooth movement (2, 14, 15). Since bone remodeling is controlled by a balance between RANK-RANKL binding and OPG production, it is important to elucidate the cellular responses in tension and pressure areas of the PDL during orthodontic tooth movement. In this context, this study was designed to evaluate expression of OPG and RANKL in GCF at tension (mesial) and pressure (distal) sides at baseline, 1 hour (h), 24 h, 168 h and 1st month after application of continuous orthodontic force.

## METHODS

Eighteen canine teeth of 9 adults (5 female, 4 male; age range 17.5-18.9 yrs) with different angle classifications were selected to participate in this study according to the following criteria: (1) being systemically healthy, (2) no use of any anti-inflammatory agents, antibiotics, immunosuppressives, or systemic contraceptives in the past six months and during the study period, (3) no evidence of periodontal bone loss in radiographs, (4) no history of smoking, (5) healthy periodontal tissues determined by clinical periodontal assessments including plaque index (PI), gingival index (GI), bleeding on probing (BOP), probing depth (PD), and clinical attachment level (CAL), (6) orthodontic treatment requiring first premolar extraction and canine distalization. All participants received periodontal prophylaxis, including scaling and polishing before the collection of GCF. The participants' rights were protected, and written informed consents were obtained according to the Gazi University Ethical Committee Board.

### Orthodontic treatment planning and sample collection

The orthodontic treatment planning required extraction of first premolars followed by distalization of canine teeth. Four weeks was waited after extraction before orthodontic force application as the healing process after extractions would change the quality and quantity of GCF. Orthodontic brackets were placed on maxillary canines (Omni Roth, GAC International Inc, Bohemia, NY) and distalization forces were applied at distal direction on the segmental 0.016 × 0.022-inch stainless-steel archwires by using sentalloy closed coil springs with eyelets generating a continuous force of 200 g. The forces were verified by a calibrated orthodontic force gauge. GCF from each tooth was sampled from mesial and distal sides at baseline (pre-treatment observation period), 1h, 24 h, 168 h and 1st month after orthodontic force application. During the examination periods, no reactivation of orthodontic force was performed. All participants maintained good oral hygiene throughout the study, and there was no apparent change in PI, GI, PD or BOP at any time or at any side.

### GCF sampling

GCF was sampled separately from the mesial and distal gingival crevices of each canine, where the orthodontic forces were applied. Supragingival plaque was removed in conjunction with a record of the PI. The teeth were gently air dried and GCF was collected after isolating the area with cotton rolls, drying the teeth and adjacent marginal gingival with air. The paper strips (Periopaper-ProFlow Inc, New York) were inserted for 30 seconds into the buccal crevice to a level of 1 mm below the gingival margin. After removing the first strip and waiting for one minute, a second strip was placed at the same side for another 30 seconds. Strips contaminated by saliva or blood were excluded from the sampled group. The paper strips from mesial and distal sides of each tooth were sealed in polypropylene containers separately. To determine the amount of GCF, an electronic scale (Precisa 62 A, Precisa instruments Ag, CH-Dietikon, Switzerland) was used for weighing the paper strips before and immediately after the collection. The difference between the two weights gave the volume of fluid collected, assuming a specific gravity of approximately 1. Each sample was stored at -70°C until being assayed.

### OPG determination

OPG levels were determined by using a commercial ELISA kit (Human Osteoprotegerin ELISA, BioVendor, Heidelberg, Germany) according to the manufacturer's instructions. In human OPG ELISA, the standards and samples (1:3 dilution) were incubated with a mouse monoclonal anti-human OPG antibody coated in wells. After washing, biotin-labeled polyclonal anti-human OPG antibody was added and incubated with captured OPG. After adding conjugate and following stop solution, the absorbance values (optical densities=OD) were measured spectrophotometrically at a wavelength of 450 nm, and the results were evaluated quantitatively according to the ODs of standard OPG concentrations with regression-correlation analysis by using a computer based statistics program called Microsta. Data was determined in picomole (pmol), and calculation of the concentration in each sample was determined by dividing the amount of OPG by the GCF volume of each sample (pmol/l).

### RANKL determination

RANKL levels were determined by using a commercial ELISA kit (sRANKL ELISA, BioVendor, Heidelberg, Germany) according to the manufacturer's instructions. Strips were eluted by centrifugation (5000 x g, 4°C, 6 min) in 300 ml of solution containing 150 mM Tris, Pepstatin A, leupeptin, amastatin and antipain dihydrochloride. ELISAs were used for quantitative detection of human total sRANKL (free and OPG-bound RANKL) and OPG (BioVendor GmbH, Heidelberg, Germany). In human total sRANKL ELISA, calibrators and samples (1:100 dilution) were incubated in wells together with the excess of recombinant OPG. The sRANKL/OPG complex appeared during the two-hour incubation step and was captured by the immobilized anti-sRANKL monoclonal antibody. After washing, horseradish peroxidase-labelled anti-OPG polyclonal antibody was added and incubated in the wells. After adding conjugate and following stop solution, the absorbance values (OD) were measured spectrophotometrically at a wavelength of 450 nm, and the results were evaluated quantitatively according to the ODs of standard RANKL concentrations with regression-correlation analysis by using a computer based statistics program called Microsta. Data was determined in picomole (pmol), and calculation of the concentration in each sample was determined by dividing the amount of RANKL by the GCF volume of each sample (pmol/l).

### Statistical evaluation

Statistical evaluation was performed by SPSS for Windows version 10.0 (Chicago, IL, USA). The average of the clinical parameters (PI, GI, BOP, PD and CAL), and the receptor levels were calculated for each subject. A 2-way repeated measures ANOVA was used to examine the effects of side (mesial/distal) and measurement time on OPG, RANKL and OPG/RANKL ratio. Greenhouse-Geisser adjustment was used to justify the assumption of sphericity. A Bonferroni test was used for pairwise multiple comparisons after any repeated-measures ANOVA that showed significance. A p value < 0.05 was considered as statistically significant.

**RESULTS**

The clinical indices of subjects were recorded at baseline, and the mean and standard deviation of PI, GI, PD and BOP were measured as 0.42 ± 0.01 mm, 0.41 ± 0.12 mm, 1.96 ± 0.16 mm, and 19.61 ± 0.01 %, respectively. No signs of periodontal destruction were observed in any subject during the study.

Tables 1 through 3 demonstrate the mean ± SD, minimum and maximum values of GCF amount, and concentration values of OPG and RANKL, respectively. Results indicated that the concentration of OPG demonstrated a rise in the mesial side at 1 h, while there was a decrease in the distal side, declaring a statistically significant difference (p = 0.019, Table 2). The concentration of RANKL did not reveal significant changes, indicating no significant effect of side and time (Table 3).

The total amount of OPG and RANKL at mesial and distal sides were similar, no significant effect of side and time on the total amounts were found, as illustrated in Figures 1 and 2 respectively.

OPG/RANKL ratio was shown in Figure 3. There was no significant effect of side and time on the OPG/RANKL ratio. An increased OPG/RANKL ratio in 1 h was followed by a decrease at 24 h, and an up-regulation was detected at 1 month.

**Table 1.** Gingival crevicular fluid amount at mesial (tension) and distal (pressure) sides (mg).

Time	Mesial side		Distal side		p
	Mean ± SD	Min-Max*	Mean ± SD	Min-Max*	
Baseline	1,5E-03 ± 0.0	2,1E-04-2,9E-03	9,7E-04 ± 0.0	6,6E-04-1,3E-03	ns
1 st hour	9,7E-04 ± 0.0	7,4E-04-1,2E-03	1,2E-03 ± 0.0	8,4E-04-1,5E-03	ns
24 th hour	9,3E-04 ± 0.0	6,6E-04-1,2E-03	1,3E-03 ± 0.0	8,3E-04-1,8E-03	ns
168th hour	1,3E-03 ± 0.0	7,3E-04-1,9E-03	9,5E-04 ± 0.0	6,2E-04-1,3E-03	ns
1 st month	1,0E-03 ± 0.0	6,8E-04-1,3E-03	9,5E-04 ± 0.0	6,5E-04-1,2E-03	ns

\* 95% confidence interval; ns, non-significant.

**Table 3.** Concentration values of RANKL at mesial (tension) and distal (pressure) sides (pmol/l).

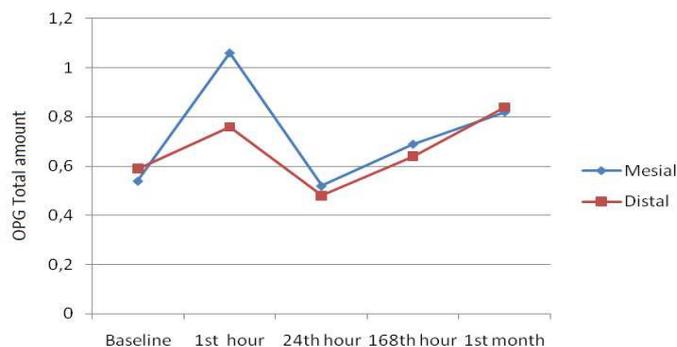
Time	Mesial side		Distal side		p
	Mean ± SD	Min-Max*	Mean ± SD	Min-Max*	
Baseline	1.11± 0.64	0.60 – 2.61	1.19± 0.71	0.36– 2.35	ns
1st hour	1.03± 0.69	0.57 – 2.72	0.83± 0.30	0.28– 1.21	ns
24th hour	1.04± 0.51	0.43 – 2.15	0.96± 0.55	0.31– 1.92	ns
168th hour	0.90± 0.44	0.28 – 1.55	1.29± 1.09	0.56– 3.69	ns
1st month	0.98± 0.32	0.49 – 1.33	1.04± 0.50	0.49– 2.08	ns

\* 95% confidence interval; ns, non-significant.

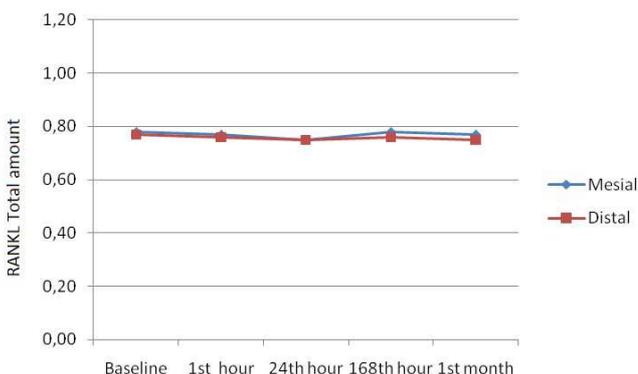
**Table 2.** Concentration values of OPG at mesial (tension) and distal (pressure) sides (pmol/l).

Time	Mesial side		Distal side		p
	Mean ± SD	Min-Max*	Mean ± SD	Min-Max*	
Baseline	0.81 ± 0.45	0.30 – 1.50	0.85± 0.53	0.19- 1.91	ns
1st hour	1.35 ± 1.13	0.29 – 3.27	0.78± 0.50	0.23- 1.67	0.019*
24th hour	0.70 ± 0.40	0.24 – 1.32	0.62± 0.33	0.18- 1.11	ns
168th hour	0.78 ± 0.46	0.15 – 1.41	1.09± 0.86	0.28- 2.62	ns
1st month	0.81 ± 0.35	0.58 – 1.64	0.97± 0.77	0.37- 2.76	ns

\* 95% confidence interval; ns, non-significant; \*p<0.05.



**Figure 1.** Total amount of OPG at mesial (tension) and distal (pressure) sides (pmol).

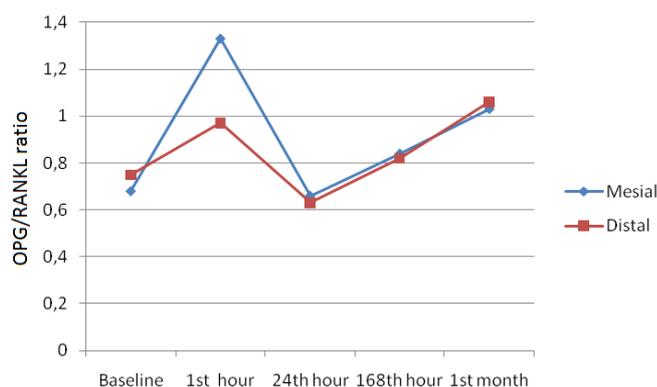


**Figure 2.** Total amount of RANKL at mesial (tension) and distal (pressure) sides (pmol).

**DISCUSSION**

Investigations identified that mechanical stimulus induces connective tissue remodeling through the up-regulation of cells in PDL, in which alveolar bone resorption and apposition is triggered. OPG and RANKL are cytokines regulating osteoclastogenesis; RANKL is a pro-resorptive factor, while OPG is a powerful osteoprotective agent (7). Orthodontic tooth movement is facilitated by bone resorption in pressure areas on the side toward which the force is directed, and deposition at the tensile areas in the contralateral position (1, 6).

In the current study, changes in OPG and RANKL levels between the tension (mesial) and pressure (distal) sides of teeth during early phase of orthodontic movement were assessed to evaluate whether the different stresses exerted on the periodontium are reflected by differences in GCF composition. Gianelly (16) stated that excessive forces compressed the roots and caused undermining resorption. It was postulated that intermittent forces produced an increase in deposition of alveolar bone, whereas continuous loading was unable to stimulate the remodeling process (17).



**Figure 3.** OPG /RANKL ratio at mesial (tension) and distal (pressure) sides.

Sentalloy closed coil springs delivering consistent forces due to their superelasticity, were used in this study in order to evaluate the effect of continuous forces in the local tension and pressure side environments. No significant elevations of OPG and RANKL were observed throughout the study, which was in accordance with a previous finding declaring that intermittent forces have greater effects in cellular activities than continuous forces (18). The only significant difference was found in the concentration of OPG, showing an increase in mesial side, and decrease in distal side, just after application of continuous force. Thus, we thought that the production of OPG is regulated differentially at tension and pressure areas as an acute response to force. Because of the less activity of RANKL at both sides in this study, it was concluded that the initial continuous force might be too heavy for the experimental period and that decreased the cellular activity.

Previously, Nishijima et al. (2) emphasized that compression force of 250 g applied with an elastomeric chain caused a significant increase in the secretion of RANKL, and decrease in OPG levels at 24 hours in GCF. Lately, Toygar et al. (19) investigated the effect of continuous force application with coil spring of 150 g on the levels of OPG in GCF, and reported that OPG concentrations in distal sides were decreased in a time-dependent manner which was in contrast to our findings. The inconsistency might be caused by the differences in study design, force magnitude, force deactivation, local response of bone or the age differences. Recently, it was found that, OPG expression was reduced after 1 h and RANKL expression was increased after six hours with compressive forces (20).

An increased OPG concentration and OPG/RANKL ratio was found in the tension side, while there was a decreased OPG concentration in the pressure side at 1 h. According to the blood flow theory related to the biologic control of orthodontic tooth movement, stimulus for cellular differentiation is produced by alterations in blood flow through the PDL in which blood flow is decreased in the compression site while it is increased in the tension side (21). These alterations in blood flow might have created changes in the chemical environment and provide differences between tension and compression sites as an early response to the mechanical force. The total amount of RANKL followed almost a linear pattern at all time intervals. On the other hand, Nishijima et al (2) found RANKL concentration maximum at 24 h in GCF of compression sides. The conflicting results may be related to the different force mechanics. When a continuous force was applied, the repair of PDL is prevented, but a period for regeneration and repair of PDL is created by intermittent forces (18). Up to a certain level of force duration or stress, the reactions occur with cell proliferation but beyond a certain level of stress or duration, destruction of cells occurs and undermining resorption become significant (22). Van Leeuwen et al. (23) discovered that continuous force application induced a constant respond in opposition to intermittent force application in PDL. Since a continuous force was applied in this study, the up-regulation of OPG after 24 h at both sides may be related to the hyalinized zones in which the cells can not differentiate into osteoclasts and no bone resorption take place for about 2-4 weeks (22). Additionally, the levels of RANKL did not change significantly, at both sites by continuous force application which may be related to the constant force magnitude.

The ratio of OPG concentration to that of RANKL was none significantly higher in mesial side at first hour compared to that in distal, which might indicate the differential regulation of OPG and RANKL in PDL cells. The increased ratio is due to a reduction in the recruitment of osteoclasts and the decreased ratio indicates an increase in osteoclastic bone resorption (24). Similar to our results, Tan et al. (24) observed higher OPG/RANKL ratio at tension sides which was decreased at first week and increased over time till the 15 days of examination period. In our study, after a sharp increase at first hour, there was a slight decrease through one week which was followed by an increase at first month. These support that tooth movement was related to the differential regulation of OPG and RANKL in PDL.

Analysis of GCF samples may help clinicians in understanding biochemical processes associated with tooth movement and could be beneficial in diagnosis, therapeutic choices, monitoring mechanical efficiencies or stabilization of the cellular activities. Further studies are needed to understand more fully the role of OPG/RANKL system in bone remodeling during orthodontic tooth movement by investigating effects of different force magnitudes and duration.

## CONCLUSION

Continuous orthodontic force evoked changes in the expression of osteoclastogenesis-inhibiting activity of PDL cells in mesial and distal sides just after force application indicating acute reaction. Due to the less active osteoclastogenesis-supporting activity with continuous force throughout the study, application of lighter forces or intermittent forces may provide greater cellular activities for regeneration of PDL.

## Conflict of Interest

No conflict of interest was declared by the authors.

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