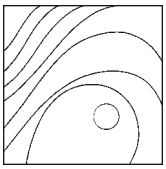
Comparison of Expression Levels of RANKL and Interleukin-17A in Male and Female Orthodontic Patients With and Without Appliances



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During orthodontic treatment, mechanical forces may change the components of gingival crevicular fluid (GCF), which reflects the cellular response in the periodontium. The objective of this study was to investigate the expressions of receptor activator of nuclear factor-kappa beta ligand (RANKL) and interleukin-17A (IL-17A) in GCF in subjects with or without orthodontic appliances, the correlation between them, their gender predominance, and their relationship with treatment time. GCF was collected from 72 young people to detect the expressions of RANKL and IL-17A. Cytokine analysis was done with the help of enzyme-linked immunosorbent assay. Results showed that the expressions of RANKL and IL-17A were higher in the treatment group than the nontreatment group ($P < .05$) and higher in males than in females ($P < .05$). Their increments were well correlated with each other ($r = 0.788$, $P = < .01$) but were not time dependent. The authors concluded that these proteins increased in the treatment group and more in the young male orthodontic patients than in the female patients. The proteins were well correlated with each other. (Int J Periodontics Restorative Dent 2015;35:e28–e34. doi: 10.11607/prd.1886)

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The extended physiologic procedures underlying orthodontic tooth movement (OTM) are the elaboration of the body's complexities. OTM is characterized by the various steps of remodeling in and around the dental and periodontal tissues. It is initiated by bone resorption and bone deposition by virtue of the mechanical forces induced by brackets and the respective orthodontic wires. Depending on the physical characteristic of the force applied, the periodontal ligament's (PDL's) vascularity and the blood flow are increased, followed by the release of various neurotransmitters, cytokines, growth factors, and colony-stimulating factors. There is a period of increase in cellular activities following the PDL width changes when orthodontic force is applied. These molecules bring about various responses in favor of the microenvironment. Orthodontic force is a highly sophisticated process as it changes the mechanical force into molecular events (signal transduction) and OTM.

After the activation of the orthodontic appliance, the patient experiences a mild aching sensation

that persists for 2 to 4 days as the events of acute inflammation prevail. The antigenic stimulation of the naive CD4+ cells give rise to the Th17 cells. This produces interleukin-17A (IL-17A), which is also produced by γδTCR cells, natural killer (NK), and natural killer T (NKT) cells,[1,2] induced by IL-6, IL-1, and transforming growth factor-beta (TGF-β), and stabilized by IL-23.[3,4] IL-17A is responsible for various autoimmune diseases,[5–7] neutrophil localization, triggering proinflammatory signals, and activating a proinflammatory program of gene expression.[8–10] It even activates the innate immunity cells that in turn activate the nuclear factor-kappa beta (NF-κβ) pathway through its proximal signaling pathways after the onset of inflammation.[5,6,11–15]

Receptor activator of NF-κB (RANK) is a key membrane protein that is expressed on the surface of osteoclasts and dendritic cells and is involved in their activation upon ligand binding. Receptor activators for NF-κB ligand (RANKL), which is a member of the tumor necrosis factor (TNF) ligand family, is synthesized by osteoblasts and contributes its lifetime to promoting osteoclast differentiation and its activation.[8,12] The binding of RANKL to the RANK leads to the rapid differentiation of hematopoietic osteoclast precursors to the mature osteoclasts. RANKL, IL-1, and IL-17A are responsible for the survival of osteoclasts, and they can induce and sustain RANK activity.[16,17]

The aim of this study was to evaluate expressions of RANKL and IL-17A in an orthodontic treatment group (with appliance) and nontreatment group (without appliance) comprising young male and female participants. The authors learned whether any correlation exists between these proteins and innovatively studied the gender predominance of these two proteins (IL-17A and RANKL) and their relation with increasing orthodontic treatment time.

Method and materials

Participants

A total of 72 healthy adolescents and young adults (ranging from 14 to 28 years of age with a mean age of 21 years) with probing depths < 2 mm were included in the study. They were selected from the Department of Orthodontics, Wuhan Union Hospital, Wuhan, Hubei Province, People's Republic of China. Patients who had periodontal disease or moderate to severe gingivitis, diabetes, had used anti-inflammatory medication within the previous 30 days, had a history of bleeding problems, used tobacco, had caries lesions or poor oral hygiene, and had received antibiotics within the previous 6 months were excluded from the study. Plaque Index and Gingival Index were recorded at the initial and recall appointments. For all participants, the Plaque Index was recorded as "0" at the baseline and recall appointments. The Gingival Index was between 0.1 and 1.0 in both the baseline and recall appointments. All participants underwent oral prophylaxis and signed a consent form.

The participants were divided into two groups: Group A (treatment group) comprised 48 adolescents and young adults (24 males, 24 females). The group was further divided into three groups equally composed of males and females. The sample collection from the first group was within 3 to 5 months from the start of the treatment, the second group was within 6 to 8 months, and the third group within 9 to 11 months. The samples were divided and collected from the patients wearing fixed orthodontic appliances at different points of time between 3 and 11 months to analyze whether the increase in the treatment time had any correlation effect on the levels of expression of the selected proteins. This time frame was selected for sample collection, because all patients were being treated by the Mclaughlin Bennett and Trevisi (MBT) appliance technique. The independent variable in this study was the MBT technique's canine laceback (canine distalization), which is usually exercised after the second orthodontic appointment (2 to 3 months).

Group B was composed of 24 adolescents and young adults (12 males, 12 females) without appliances. Participants in group B were candidates for orthodontic treatment and, therefore, served as the positive controls.

This allocation represented the wide range of age groups and achieved 90% power to detect a pattern of RANKL and IL-17A. There was no substantial reduction in the power of statistical analysis with this pattern of allocation.

Gingival crevicular fluid sampling

The gingival crevicular fluid (GCF) was collected by Millipore filter membranes (pore size 0.22 mm; Millipore) from the mesial, medial, and distal sides of the maxillary right canine being distalized with 0.25-inch ligature wire (independent variable). All patients in the treatment group either had maxillomandibular protusion or anterior crowding. All patients required extraction of the first premolars and distalized canine (dependent variable, treatment outcome variable) to treat the malocclusion. MBT brackets were bonded in all patients, and the canine laceback technique was exercised. The experimental tooth was gently washed with water, and the gingival area was isolated with cotton rolls and gently dried with an air syringe. A first Millipore polyvinylidene fluoride (PVDF) strip was very carefully inserted 1 mm into the sulcus and left in situ for 15 seconds. After removal of the first strip, a second PVDF strip was similarly inserted in the other two sites for 15 seconds. In the event of visible contamination with blood, the strips were discarded. After removal from the sulcus, each of the strips was placed in a Periotron 700100 (Oraflow) for GCF volumetric measurement. Within 60 minutes, proteins in the three strips were eluent in 500 µL isotonic phosphate-buffered saline, pH = 7.4, without detergent and protease inhibitors, and immediately frozen at –20°C until the day of the analysis. The number of freeze-thaw cycles was kept to a minimum. The recovery of most proteins was satisfactory and reproducible.

Analysis of cytokine production

All samples were analyzed for RANKL and IL-17A with the help of Sigma-Aldrich's 3-ethylbenzothiazoline-6-sulfonic acid liquid substrate system for enzyme-linked immunosorbent assay (ELISA) and the Human IL-17A Mini ELISA Development Kit (900-M84, PeproTech), respectively. The microcentrifuge tubes with strips kept at the collar to elute all the GCF components completely were centrifuged at 2,000 g for 5 minutes. After the strips' removal, the supernatant was divided into two aliquots for the determination of each biologic compound. The assays were carried out in accordance with the manufacturer's instructions, and the levels of the biochemical compounds were reported as the optical density (OD) values.

Statistical analysis

The one-way analysis of variance, using SPSS version 20 statistical analysis software (SPSS) in all samples was done to compare the different variables. $P < .05$ was considered significant. The Pearson correlation test was significant at the .01 level. The value of r (coefficient of correlation) obtained was compared to the critical value table for 48 (undertreatment) samples.

Results

Intergroup comparisons of IL-17A in males and females

The results showed that the expression level of IL-17A was much higher in group A, which strongly denoted the underlying process of inflammation (Fig 1); $P = .00$.

Intergroup comparisons of RANKL in males and females

The results showed the expression of RANKL significantly increased in the treatment group (group A), similar to IL-17A (Fig 2); $P = .00$.

Intragroup comparisons of IL-17A

A comparison was done between the male and female patients in the same group. It showed a significant increase in the expression of IL-17A in the males. This signified that the cytokine remained elevated in the treatment as well as the nontreatment "male" group significantly more than that of the female counterparts. The underlying inflammation following the orthodontic treatment was significantly higher in men (Fig 3); $P = .01$.

Intragroup comparisons of RANKL

A similar result comparing expression of RANKL between both sexes of groups A and B justified the fact that the process of ongoing osteo-

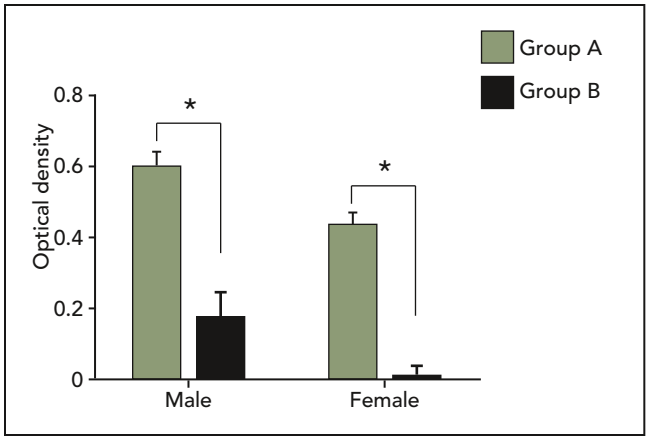

Fig 1 *Intergroup comparison of IL-17A. The males and females of the treatment group (group A) show a significantly higher (P < .05)* level of IL-17A than the males and females of the nontreatment group (group B).*

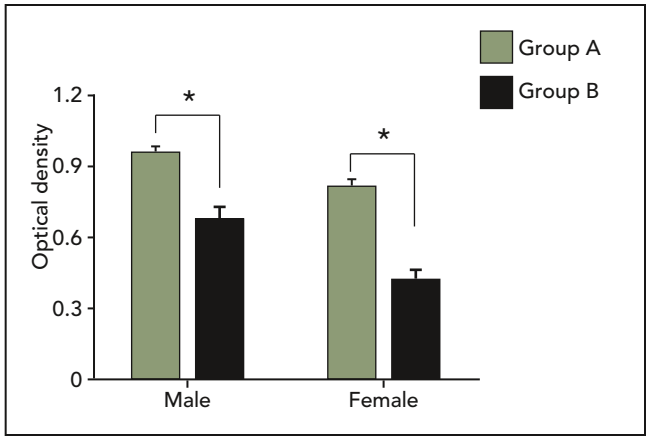

Fig 2 *Intergroup comparison of RANKL. The males and females of the treatment group (group A) show significantly higher (P < .05)* levels of RANKL than the males and females of the nontreatment group (group B).*

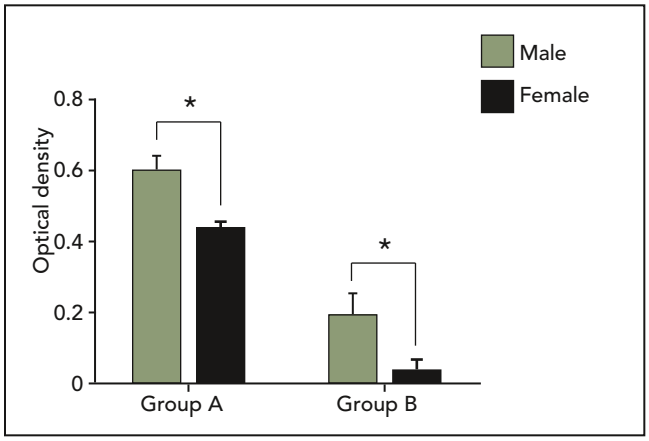

Fig 3 *Intragroup comparison of IL-17A. The males of both the treatment group (group A) and the nontreatment group (group B) show significantly higher (P < .05)* levels of IL-17A than the females of group A and group B.*

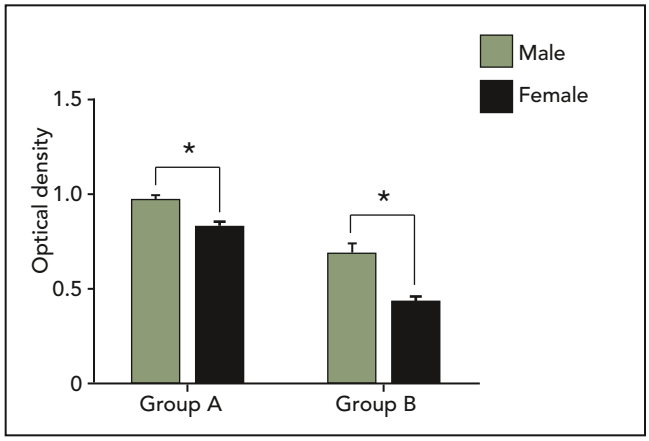

Fig 4 *Intragroup comparison of RANKL. The males of both the treatment group (group A) and the nontreatment group (group B) show significantly higher (P < .05)* levels of RANKL than the females of group A and group B.*

clastogenesis was maximum in the male patients after the application of orthodontic forces and that this cytokine remains more elevated in males of the nontreatment group than their female counterparts. (Fig 4); $P = .00$.

Pearson correlation test

There was an absolute positive correlation between IL-17A and RANKL ($r = 0.788$, $P = < .01$), n = 48. The r value was higher than the critical table value for 48 samples at the significance level of .01 (Fig 5).

Increments in the levels of RANKL and IL-17A with treatment time

1. RANKL with time $P = .334$ ($P > .05$), not significant
2. IL-17A with time $P = .707$ ($P > .05$), not significant

Discussion

In the present study, the levels of expression of IL-17A and RANKL were monitored and were found to be correlated in the GCF among the patients being treated orthodonti-

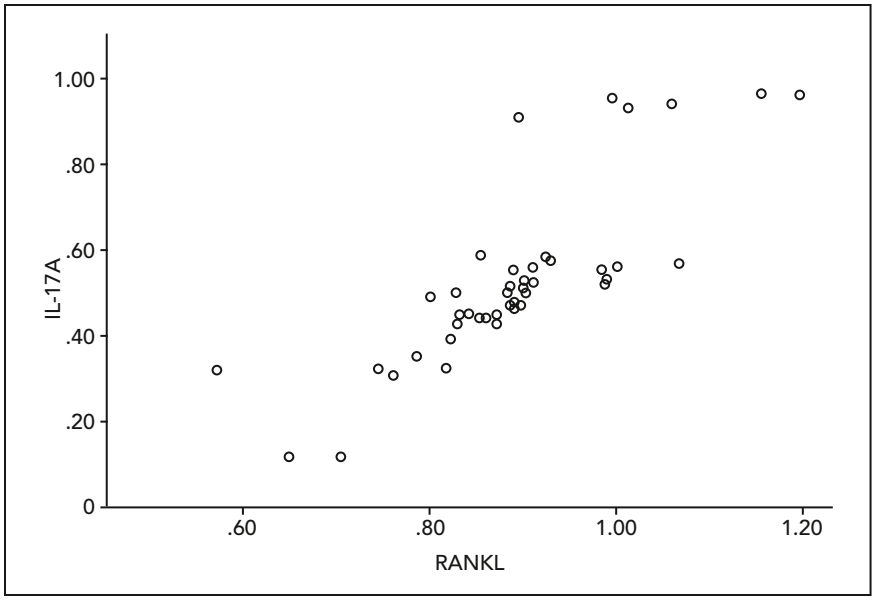

Fig 5 *Pearson correlation test between IL-17A and RANKL. The r value = 0.788, which was higher than the significance level of .01.*

cally. IL-17A activates the innate immunity cells that in turn activate its NF-κß proximal signaling pathways, which is a hallmark transcriptional factor associated with the onset of inflammation.[5,6,11–15] The expression of IL-17 and its receptors in the osteoblast-like cells occur as a result of the compressive forces that may in turn initiate the osteoclastogenesis through the expression of the RANKL in osteoblasts mitogen-activated protein kinases.[18]

The results demonstrated that the young adults with fixed orthodontic appliances had significantly higher levels of IL-17A and RANKL than the controls. The authors attributed this result to orthodontic treatment in group A. This strongly indicated the process of ongoing inflammation, osteoclastogenesis, and OTM in the treated group. IL-17A and RANKL were responsible for the elevation of the pool size of inflammatory cells and the osteoclasts in the regions where the orthodontic force was applied. These findings are in line with the previous reports of compression during orthodontic force, which increased the levels of RANKL in human periodontal ligament (hPDL)-derived cells of the GCF with 16.7-fold, strong involvement of the RANKL in the alveolar bone remodeling during the OTM,[19] the final effector of osteoclastic differentiation and function.[20] IL-17A is responsible for various autoimmune diseases[5,6] and neutrophil localization, and it triggers and activates a proinflammatory program of gene expression.[8–10] The present authors believe that in this study, in young females whose estrogen is at a higher level, the expressions of osteoclastogenesis-inducing proteins might be less. This new concept is in line with the studies that proved that estrogen inhibited or often modulated the expression of RANKL induced osteoclastic differentiation, antibone resorptive functions, fluctuations in serum markers of bone turnover,[21] and estrogen therapy showed slower rate of tooth movement.[22] Orthodontic force application after ovulation accelerates tooth movement,[21] and ovariectomy leads to significantly greater bone resorption.[23,24] In this study's comparisons, the females showed a significantly lower expression of RANKL and IL-17A. This can be attributed to the fact that females have a higher level of estrogen and, therefore, suppress the expression of these proteins.

Orthodontists want to accelerate treatment and finish their cases sooner and without compromise. With the above knowledge in hand, clinicians should be aware that it may take a little more time to finish a case with a young female patient than a male patient of a similar age because the young female harbors a high amount of estrogen and expresses a lower level of proteins

(RANKL and IL-17A), and, thus, a lower rate of OTM. The proteins RANKL and IL-17A are estrogen dependent; as a result, bone turnover is affected. Therefore, certain measures to accelerate the OTM by activating the appliance just after the estrous cycle or by the use of devices such as AcceleDent should be encouraged in young female patients. AcceleDent, invented by a professor at Columbia University, New York, NY, USA, is a fast and gentle hands-free device that is designed to accelerate the bone remodeling process that complements conventional orthodontic treatment.[25,26]

Although both of the experimental proteins increased more in the treatment group than in the control, the increment was higher in the young males. This results in more alveolar bone resorption of the young male patients, faster OTM, and less time to finish the orthodontic treatment.

If prior investigation of the interaction between the environmental factors (including the treatment) and each patient's unique genetic factors on bone biology through associated studies is done, the application of the treatment would be refined and the orthodontic force could be optimized. However, cytokine analysis can only be carried out in special cases to avoid the unwanted results of excessively slower OTM, as with patients undergoing estrogen therapies and patients taking oral contraceptives. It is also of the utmost importance to maintain good oral hygiene in all patients because it helps to limit the expression of unwanted higher levels of inflammatory cells causing inflammation and bone destruction.

Conclusions

Significantly correlated levels of IL-17A and RANKL show that the increment in one of these molecules has a significant effect on the other. The proteins are not dependent on the increase in treatment time ($P > .05$). These higher levels of IL-17A and RANKL are brought about by the orthodontic treatment procedures. These molecules also are significantly increased in young males more than in their female counterparts. This is attributed to the fact that females have higher levels of estrogen. This may also explain slower OTM during treatment in female patients than male patients of similar mean age, thus resulting in the clinician taking a longer time to complete treatment.

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