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Research paper

Cytokine profile changes in gingival crevicular fluid after placement different brackets types

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ABSTRACT

Objective: The aim of this study was to examine the relationship between bracket design and ratio of five proinflammatory cytokine, in gingival crevicular fluid (GCF), and bacterial adhesion without tooth movement influence.

Design: The sample was comprised of 20 participants, aged 11 to 15 years old (mean age: 13.3 years ± 1.03). A conventional Gemini™ metallic bracket and two self-ligating brackets, In-Ovation®R and SmartClip™, were bonded to the maxillary incisors and canines. GCF was collected using a standard filter paper strip before and 60 days after bonding. The cytokine levels (IL-12, IL-1α, IL-1β, IL-6 and TNF-α) were performed by the LUMINEX assay. The levels of the red and orange bacterial complexes were analyzed by the Checkerboard *DNA-DNA hybridization.* The data of cytokine and bacterial complexes were carried out using the non-parametric tests at 5% of significance level.

Results: Increased cytokine levels were observed. However, only the SmartClip™ group showed a significantly increased level of TNF-α ($p = 0.046$). The SmartClip™ brackets group presented higher levels of red complex bacteria.

Conclusions: The bracket design affected cytokine levels and bacterial adhesion since it was observed that the proinflammatory cytokines released in GCF to the SmartClip™ group showed an increase in the TNF-α levels associated with higher bacterial levels, which possibly represents greater inflammatory potential. Thereby, the bracket design should be considered in patients with risk of periodontal disease and root resorption.

1. Introduction

Nowadays, patients expect treatments that are effective, fast, and that do not promote damage in the teeth and periodontal tissues. In this context, many types of orthodontic brackets are commercially available for clinical use. Self-ligating brackets present some advantages in comparison with conventional brackets, such as reduced treatment time, reduced number of dental appointments, and the effectiveness of treatment (Čelar, Schedlberger, Dörfler, & Bertl, 2013; Fleming & O'Brien, 2013; Harradine, 2013). Regardless of the type, any orthodontic appliance promotes significant changes in the homeostasis of the periodontal tissues (Alfuriji et al., 2014) by the increase of dental plaque and the release of chemical mediators in the gingival sulcus (Jurela et al., 2013; van Gastel, Quirynen, Teughels, Coucke, & Carels, 2008).

Cytokines induce and maintain a chronic inflammatory response in the periodontium. Gingivitis increases blood flow, vascular permeability, and inflammatory cell migration (neutrophils and macrophages) from peripheral blood to the crevicular fluid. Subsequently, T and B-lymphocytes appear at the injury site. Host cells produce and release cytokines such as IL-1α, IL-1β, IL-6, IL-8, TNF-α, and prostaglandins (Marcaccini, Amato, Leão, Gerlach, & Ferreira, 2010; Ziegler et al., 2010). In this way, the literature has empathized the role of the cytokines in orthodontic movement (Andrade, Silva, Silva,

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Teixeira, & Teixeira, 2007; Garlet et al., 2008; Kapoor, Kharbanda, Monga, Miglani, & Kapila, 2014). In pathological conditions, these cytokines regulate bone reabsorption, which could lead to the occurrence of bone or radicular resorption (Belibasakis & Bostanci, 2012; Sims & Gooi, 2008) during orthodontic treatment (Marcaccini, Amato, Leão, Gerlach, & Ferreira, 2010; Viecilli, Katona, Chen, Hartsfield, & Roberts, 2009; Ziegler et al., 2010).

Thus, to evaluate if the bracket design induces the accumulation of bacterial plaque and promotes inflammation of the supporting tissues, our research group carried out an ample study that analyzed the periodontal indexes, bacterial behavior, and gingival crevicular fluid 60 days after bonding different types of orthodontic brackets: conventional metallic (Gemini™) and active (In-Ovation®R) and passive (SmartClip™) self-ligating brackets. Initially, the periodontal parameters and the volume of the gingival crevicular fluid were evaluated, and it was verified that the bracket design influenced the plaque index and fluid volume. In these features, the self-ligating SmartClip™ presented the worst performance (Bergamo et al., 2016). When the bacterial dynamics correlated with periodontal disease were evaluated over 60 days, a distinct contamination pattern was observed for the self-ligating brackets, which showed highest levels of bacterial species involved in periodontal disease (Bergamo et al., 2017).

On a multilevel aspect, the bonding process, as well as the bracket design, may promote changes in gingival and periodontal tissues, even in the absence of orthodontic forces. However, only a few studies have focused on the evaluation of these alterations according to self-ligating brackets.

Therefore, the aim of this study was to evaluate the cytokine levels (IL-12, IL-6, IL1-α, IL-1β and TNF-α) in the gingival crevicular fluid, and the bacterial complex profile *in situ,* before and 60 days after bonding of self-ligating and conventional brackets. The null hypothesis tested was that the bracket design does not affect the cytokine profile, orange and red complexes levels.

2. Materials and methods

The ethics committee approved the present study (research protocol number #0062.0.138.000-10). Informed consent was obtained from the patients or their parents before the study. This protocol was performed in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The sample size calculation was performed using the SPSS program SamplePower (IBM software-Statistical Package for the Social Sciences, Inc. Chicago Illinois, USA). The calculation was based on five factors: I) difference between initial and final means; II) dispersion of scores; III) sample loss; IV) alpha value of 0.05; and V) bicaudal analysis. A sample of 20 subjects per group would have 80% power.

Twenty patients referred to the Orthodontic Clinic were included. The subjects were selected according to the following exclusion criteria: history of previous orthodontic treatment, history of antibiotic therapy in the last 3 months, history of systemic medication in use, current smoker, diagnosis of systemic disease, and signs of gingivitis and/or periodontitis. Patients with severe crowding, overjet, and overbite were also excluded.

Standardized hygiene instructions were given to all patients by the same investigator. Patients were provided with a toothbrush (Professional®, Colgate-Palmolive Industry, São Bernardo do Campo, SP, Brazil) and a toothpaste (Oral-B® Pro-Saúde©, 2012 Procter & Gamble of Brazil).

2.1. Bracket bonding and debonding

All the patients received metallic brackets: two self-ligating (In-Ovation®R, Dentsply, GAC and SmartClip™, 3M Unitek, Monrovia, CA, USA) and one conventional bracket, used with elastomeric ligatures (Gemini™, 3M Unitek, Monrovia, CA, USA).

The schematic drawings of the six anterior teeth were designed to distribute the different types of brackets in the previous six teeth selected for bonding. Thus, the different brackets had been listed according to the type of the bracket and the time of debonding. The brackets were numbered from 4 to 6 with the following distribution: the number 4 matched the In-Ovation®R bracket, the number 5 matched the (Gemini™, 3M Unitek, Monrovia, CA, USA). SmartClip™ bracket, and the number 6 matched the Gemini™ bracket, removed 60 days after bonding. This random assignment also ensured that the number of each type of bracket removed, sixty days after bonding, was similar for each anterior tooth analyzed for both the left and the right side. A total of 60 brackets were removed, 60 days after bonding, 20 of each type, distributed similarly among the different dental elements.

The Transbond XT system (3M Unitek, Monrovia, CA, USA) was selected for the bonding. After bonding, a 0.014" orthodontic archwire was inserted passively.

After 60 days, the brackets were debonded and were placed into the coded sterile microtube tubes containing 150 μL of TE (10MmTris-HCl, 1MmEDTA pH 7.6) and mixture in Vortex. The brackets were removed using the sterilized pliers followed by the addition of 100 μL of 0.5 M NaOH and stored at −20° C until the *DNA–DNA checkerboard hybridization* was performed, according to Bergamo et al. (2016).

After this stage, all patients were enrolled in a corrective orthodontic treatment and received new brackets.

2.2. Gingival crevicular fluid collection

At the baseline, before the GCF collection and bracket bonding, the teeth were pumiced, washed, and dried, the areas were isolated with cotton rolls and gently dried. The GCF was collected according to Iwasaki, Haack, Nickel, Reinhardt, and Petro (2001). PerioPaper absorbent strips (PerioPaper®, Oraflow Inc., Plainview, USA) were placed into the sulcus. After keeping the strip in place for 30 s, the absorbed volume was measured with the Periotron® 8000 (Oraflow Inc., Plainview, USA). Strips with blood contamination were discarded. In order to minimize evaporation, the volume was analyzed as fast as possible. Three strips were collected from three sites on the buccal surface (mesial, central, and distal) in each tooth.

The brackets were debonded after 60 days, and the GCF collection was repeated, before debonding. The PerioPaper strips were placed in coded sterile microtubes and stored at −70° C until cytokine analysis.

2.3. Cytokines measurement

Cytokine levels of IL-12, IL-1α, IL-1β, IL-6, and TNF-α were determined in the GCF atT0 and T1 using LUMINEX® assay. A high light sensitivity human cytokine kit (HCYTOMAG-60K-05; Milipore, Bilerica, MA, EUA) was used according to the manufacturer's instructions, using the multiplexing instrument MAGpix™ (MiraiBio, Alameda, CA, USA).

The samples were individually evaluated, and the concentrations were estimated from the standard curve using a five-parameter polynomial equation using Xponent® software (Millipore Corporation, Billerica, MA, USA). The mean concentration of each biomarker was calculated, adjusted to GFC volume, and expressed as pg/mL.

Briefly, a 96-well plate was prewet with washing buffer, which was subsequently discarded, followed by the addition of microsphere magnetic beads coated with monoclonal antibodies against the five different target analytes to the wells. Samples and standards were added to the wells and incubated for two hours under gentle agitation and in darkness. The wells were washed using a magnetic manifold, and a mixture of biotinylated secondary antibodies was added. After incubation for 1 h, streptavidin conjugated to the fluorescent protein RPhycoerythrin was added to the beads and incubated for 30 min. After washing to remove the unbound reagents, sheath fluid was added to the wells, and the beads (minimum of 50 per analyte) were analyzed in the multiplex assay instrument. Samples were diluted with the diluents

from the kits. The dilution was taken into consideration when calculating the concentration of each substance with a standard curve and were prepared using the standard proteins in the kit. Enzyme-linked immunosorbent assays were run in duplicate, and mean values were used to calculate the concentrations of each marker.

2.4. Bacterial complex profile

The levels of orange and red complexes *in situ* were determined using the *DNA–DNA checkerboard hybridization* method previously describe by Bergamo et al. (2017).

The evaluated target species of orange complex bacteria was *Campylobacter rectus* (ATCC-33238), *Fusobacterium nucleatum* (ATCC-25586), *Fusobacterium periodonticum* (ATCC-33693), *Prevotella intermedia* (ATCC-25611), *Prevotella melaninogenica* (ATCC-25845), *Prevotella nigrescens* (ATCC-25261) and red complex bacteria was *Porphyromonas gingivalis* (ATCC-33277), *Tannerella forsythia* (ATCC-43037) and *Treponema denticola* (ATCC-35405).

The total amount of species in each complex was taken account to analysis.

2.5. Statistical analysis

The non-parametric Friedman and Wilcoxon test was employed. SPSS 17.0 statistical software (IBM Software-Statistical Package for the Social Sciences, Inc., Chicago, Illinois, USA) was used for data analysis with an established alpha of 0.05.

3. Results

Table 1 displays all demographic characteristics and malocclusion features of the included subjects. The mean age was 13.3 ± 1.01 and 55% were female. Regarding the malocclusion type, 36.8% of subjects were type 1 crowding (at upper arch), 47.4% were type 1 overbite, and 47.4% type 1 overjet (Tables 1 and 2).

The cytokine levels (pg/mL) of IL-12, IL-6, IL1-α, IL1-β, and TNF-α are presented in Table 3. It should be noted that no difference between bracket types were observed at baseline or at 60 days for all cytokines ($p > 0.05$).

However, TNF-α levels increased from baseline to 60 days only in the SmartClip™(self-ligating) group, while no changes overtime were observed in Gemini™(conventional) or In-Ovation®R(self-ligating) groups (Table 4).

In bacterial analyses, Friedman test pointed significantly difference only to red complex $p = 0.0074$. A statistically significant difference was observed between SmartClip™ and Gemini™ brackets by posttest ($p = 0.016$). The bacterial complex distribution among the brackets is presented in Fig. 1.

The evaluated hypothesis was rejected.

Table 1
Demographic characteristics and malocclusion feature scores.

Patient	Crowding Score	Overjet Score	Overbite Score	Sex	Age
1	1	0	1	F	13
2	2	1	1	M	14
3	0	1	2	M	14
4	1	1	2	M	12
5	1	1	3	M	12
6	2	0	0	M	15
7	2	0	2	M	14
8	0	0	1	F	14
9	0	1	1	F	14
10	1	0	1	F	14
11	1	0	0	F	14
12	0	1	3	M	14
13	0	1	1	M	14
14	0	0	1	F	13
15	1	0	1	F	13
16	2	1	2	F	13
17	0	1	1	M	14
18	1	0	0	F	12
19	1	3	3	F	12
20	1	1	3	F	11

Crowding score, Overjet Score, and Overbite Score according to criteria in Table II; F-female gender; M – male gender; Age in years.

Table 2
Malocclusion features scores.

Scores	Crowding Upper arch	Overbite Lower incisor coverage	Overjet Superior to inferior of incisor ridges
0	Less than 2.0 mm		
1	2.1 mm to 5.0 mm	Overbite < 1/3	1 mm–3.0 mm
2	5.1 mm to 9.0 mm	1/3 to 2/3 tooth	3.1 mm–6.0 mm
3	9.1 mm to 13.0 mm	2/3 tooth to full covered tooth	6.1 mm–9.0 mm
4	13.1 mm to 17.0 mm	Full covered tooth	More than 9.1 mm.
5	More than 17.1 mm	**	**

4. Discussion

Periodontal disease is a multifactorial condition that involves environmental, microbiological, and host factors. It is well known that orthodontic appliances are environmental factors involved in the complex etiology of this disease (Darveau, 2010; Socransky & Haffajee, 2005; Wade, 2013). The multilevel complex interactions involved in the disease's etiology are still poorly understood. Considering the complex scenario conducted by numerous pro-inflammatory and anti-inflammatory mediators with different properties, this study aimed to evaluate the role of the brackets' design in the release of cytokines as a host factor. Our results demonstrated the role of the bracket design in the cytokine level alteration.

The GCF is an important sample to evaluate the inflammatory condition of the periodontal tissues. *In vivo,* it is an important marker of sulcus ecology and periodontal pockets, as it contains cytokines, bacterial products, and sub-products (Goodson, 2003). As the composition and volume of crevicular fluid undergo changes associated with the inflammation of gingival tissues, their analysis is adequate to study pathological changes in humans because it can be obtained non-invasively and allows repeated collection from the same location (Lamster & Ahlo, 2007). Cytokines present in GFC during treatment provide information on cellular metabolism, periodontal health, and bone remodeling since cytokines can also modulate the differentiation and proliferation of osteoclasts (Andrade, Silva, Silva, Teixeira, & Teixeira, 2007; Kapoor, Kharbanda, Monga, Miglani, & Kapila, 2014; Marcaccini, Amato, Leão, Gerlach, & Ferreira, 2010).

IL-6, IL-1α, and IL-1-β, are known to induce the differentiation and proliferation of osteoclasts, stimulating bone resorption. During orthodontic movement, high levels of IL-6, IL-1α, and IL-1-β are identified on the compression side (Iwasaki, Haack, Nickel, Reinhardt, & Petro, 2001; Salla et al., 2012; Zainal Ariffin, Yamamoto, Zainol Abidin, Megat Abdul Wahab, & Zainal Ariffin, 2011). In the present work, orthodontic wire was kept passively in all brackets, and the collection was performed after the influence of orthodontic movement. We must emphasize that group which received In-Ovation®R did not show an increase in the periodontal index, crevicular fluid volume, nor in either the orange or red complex bacterial levels. The Gemini™ showed the lowest values of the periodontal index and orange/red complexes

Table 3
Gingival Crevicular Fluid cytokine levels (pg/mL) in the three different brackets.

Cytokine	Brackets	T0 Mean ± SD	T0 Median	T1 Mean ± SD	T1 Median	p
IL-12	SmartClip™	45.88 ± 75.62	28.55	48.76 ± 67.06	22.8	
	Gemini™	49.06 ± 76.93	23.90	78.86 ± 145.02	32.40	0.76
	In-Ovation®R	23.59 ± 20.31	13.45	76.59 ± 122.95	33.20	
IL-1α	SmartClip™	434.55 ± 309.73	459.50	860.10 ± 1220.08	380.0	
	Gemini™	588.73 ± 568.34	421.0	851.32 ± 885.89	679.0	0.39
	In-Ovation®R	471.98 ± 315.92	400.0	534.94 ± 520.95	329.0	
IL-1β	SmartClip™	4.58 ± 7.00	2.15	3.96 ± 6.63	1.7	
	Gemini™	18.02 ± 51.48	1.35	4.21 ± 7.34	1.8	0.78
	In-Ovation®R	4.02 ± 6.97	1.69	2.91 ± 5.12	1.1	
IL-6	SmartClip™	11.86 ± 16.10	3.8	22.68 ± 56.02	4.0	
	Gemini™	5.23 ± 7.29	1.9	7.15 ± 13.33	3.05	0.67
	In-Ovation®R	11.47 ± 16.98	4.5	8.22 ± 10.32	3.35	
TNF-α	SmartClip™	3.32 ± 4.15	1.75	4.62 ± 3.83	3.9	
	Gemini™	3.16 ± 3.79	1.35	4.61 ± 3.80	3.3	0.10
	In-Ovation®R	5.55 ± 12.41	1.60	4.77 ± 7.88	2.5	

T0- before bonding; T1- 60 days after bonding; ± SD – Standard deviation; p – Friedman test.

Table 4
Comparing between each bracket before bonding and 60 days after bonding.

Cytokine	p-value for SmartClip™	p-value for Gemini™	p-value for In-Ovation®R
IL-12	0.351	0.161	0.199
IL-1α	0.370	0.263	0.911
IL-1β	0.940	0.672	0.243
IL-6	0.737	0.588	0.179
TNF-α	0.046*	0.091	0.341

p Wilcoxon test; * statistically significant difference.

levels. The SmartClip™ presented the highest levels for all parameters analyzed. This could explain our results, in which alterations were not observed in IL-6, IL-1α, and IL-1-β.

IL-12 is associated with inhibition of bone resorption and reduction of orthodontic movement by inhibiting osteoclastogenesis. IL-12 is a pro-inflammatory cytokine that induces differentiation of T cells into interferon-γ and T-helper 1 cells (Shaddox et al., 2011). The response to the bacterial endotoxin is different for each pathogen species, and certain bacterial lipopolysaccharides can increase the expression of pro-inflammatory cytokines like IL-12. (Shaddox et al. 2013) IL-12 act with TNF-α in host defense, but also may inhibit excessive TNF-α. In this study, the levels of IL-12 did not change, in spite of the increase of the TNF-α, and red complex levels. The mild inflammation identified in this sample did not affect the IL-12 levels.

The host response is represented by the reaction of the periodontal tissues to bacterial products by secreting cytokines and promoting the remodeling of bone tissue (Kitaura et al., 2014; Ren, Hazemeijer, de Haan, Qu, & de Vos, 2007). In the present study, wenoted that a significantly increased level of TNF-α occurred at T1 for the SmartClip™ bracket group. TNF-α is a cytokine secreted by lymphocytes, fibroblasts, leukocytes, and epithelial cells of the gingival tissue and has a key role in the inflammatory process by inducing bone resorption by over-expression of the nuclear factor κB (RANKL) (Wei, Kitaura, & Zhou, 2005; Kim, Park, Park, Lee, & Kang, 2013). Its

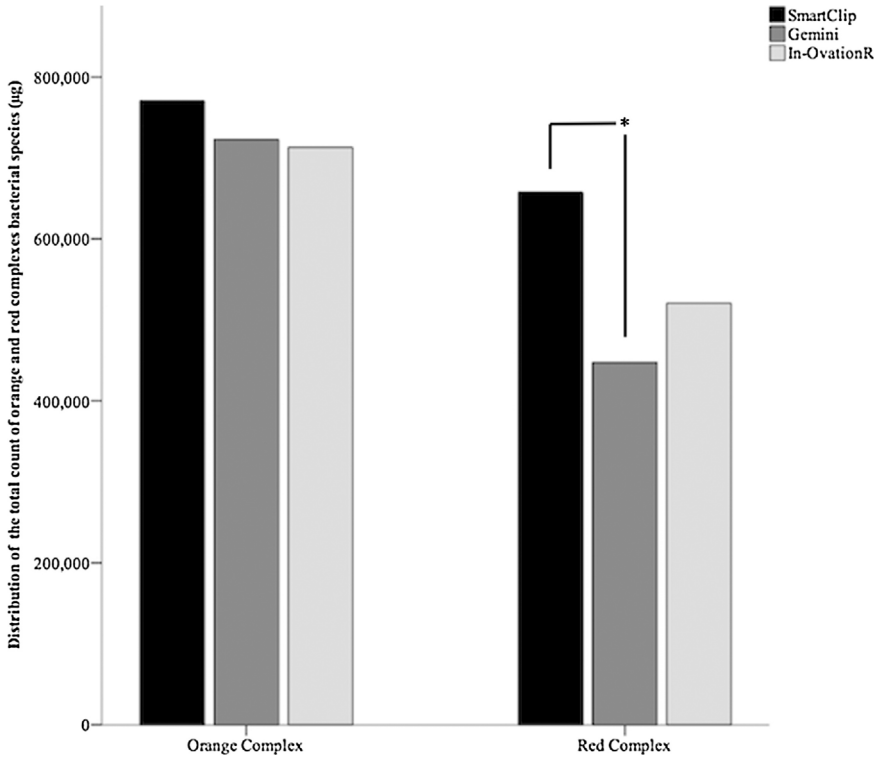

Fig. 1. Total count of bacterial species (µg) belonging orange and red complexes.

secretion is increased for stimulating the bacterial endotoxin, a component of the cell wall of Gram-negative microorganisms in the red complex (Nelson-Filho et al., 2011) that could induce inflammation (Kajiya et al., 2010) by adhering to the surface of the brackets *in situ*. It is interesting that only the red complex levels were different between the brackets designs. In fact, there was a correlation between the production of inflammatory cytokines and the presence of red complex bacteria species (Andrade, Silva, Silva, Teixeira, & Teixeira, 2007). Also, cytokines have an important role in host defense and immune system regulation, and they are influenced by periodontal parameter alterations. As previously described by Bergamo et al. (2016), the volume of the GCF and plaque index increased, at 60 days after bonding to the SmartClipTM bracket group, which could result in gingival inflammation and could increase the pro-inflammatory cytokine levels, which was observed in the TNF-α levels in this study.

Thereby, from a clinical point of view, it was observed that the levels of pro-inflammatory cytokines released in the crevicular fluid was similar in the Gemini™ (conventional) and In-Ovation®R (self-ligating) brackets. However, the SmartClip™ (self-ligating) showed an increase in the TNF-α levels in the crevicular fluid, which possibly represents greater inflammatory potential. It is reasonable to assume that many factors, acting alone or in combination, contribute to the health of the oral environment during orthodontic treatment.

Considering the relevant influence of the wide variety of bracket designs on the dental plaque accumulation and periodontal response inflammatory trigger, additional studies investigating a large sample model with orthodontic movement are needed.

5. Conclusions

The brackets design affected the cytokine and bacterial complexes levels since the SmartClip™ bracket presented significant alterations. The highest levels of the pathogenic bacteria from the red complex was observed to this bracket simultaneously with an increased concentration of TNF-α.

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