

Full Length Research Paper

Detection of dentin antigenic fractions by salivary immunoglobulin G in patients undergoing orthodontic treatment

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This study aimed to partially characterize the antigenic fractions of human dentin extract, and to evaluate the anti-dentin antibodies levels in patients undergoing orthodontic treatment. Two dentin extract fractions (FI and FIIa) were used to analyze the saliva samples from three groups of subjects. One group had 13 subjects who presented radiographic signs of mild to moderate root resorption 12 months after starting orthodontic treatment (T12); saliva samples taken prior to treatment served as controls (T0). The other groups had 10 subjects with radiographic signs of mild to severe root resorption up to 48 months after orthodontic therapy (PT); 10 individuals not undergoing orthodontic treatment were selected as controls (ST). Western blot analysis revealed the presence of dentinal fractions of approximately 35 to 70 kDa in (T12) and (PT) but not in the control samples. In immunoenzymatic assays, the anti-FI salivary IgG levels were significantly higher in T12 and PT groups than in the controls ($P < 0.05$). Our results demonstrate that dentinal fractions may be presented to the immune system and detected by salivary IgG. The levels of anti-dentin antibodies may remain elevated even years after finishing of orthodontic therapy.

Key words: Root resorption, dentinal antigens, humoral response, enzyme-linked immunosorbent assay (ELISA), Western blot.

INTRODUCTION

External apical root resorption (EARR) is an immunopathological manifestation of inflammatory origin that presents high prevalence (39 to 99%) in patients undergoing orthodontic treatment (Apajalahti and Peltola,

2007; Dudic et al., 2008). This process has consequence the irreversible shortening from apex root. Although in most cases the root resorption is mild and minor clinical importance, moderate (lesser than 2 mm) to severe

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(greater than 2 mm) root resorption has been reported to occur at a frequency of 10 to 20% (Brin et al., 2003; Marques et al., 2010), representing a serious problem for orthodontists. The combination of several mechanical and biological risk factors, reported in the literature as: extensive tooth movement, movement type, orthodontic force magnitude, duration and type of force, individual susceptibility, anatomical factors, genetic influence, systemic factors (Sameshima and Sinclair, 2001; Al-Qawasmī et al., 2003; Segal et al., 2004; Artun et al., 2009), may predispose EARR. However, the destruction of the cementoblastic layer, that protect the tooth root, is the local factor required for the initiation of (Consolaro, 2005). Although the mechanisms of induction and regulation of this pathology are not yet completely understood, some results have indicated the potential of dentin to induce inflammatory and immune response in the root resorption process. Dentin contains numerous non-collagenous proteins (NCP) and signaling molecules usually hidden, trapped in their mineralized matrix. These dentin constituents, that seem to be multifunctional, can be exposed and released in consequence of local injury as from orthodontic movement (Butler et al, 1998; Silva et al., 2004).

The exposure of the dentin *in vivo* is, frequently, accompanied by inflammatory cell infiltration on periodontal tissues and by signals and cytokines production, for activation and differentiation of clasts, responsible by resorption of root surface (Bassaran et al., 2006). It is possible that clasts dissolve the mineralized matrix, endocytose, transport and continuously release dentin molecules in the tooth/periodontium microenvironment during the resorption process (Nesbitt 1997).

These components may act as chemotactic factor (Ogata et al., 1997) and activation factor of inflammatory cells (Lara et al., 2003) and resorptive cells (Nakagawa et al., 2000). Additionally, previous studies already have established the immunogenic potential of dentin and confirmed the presence of anti-dentin antibodies (autoantibodies), by using a model of root resorption in dogs and mice (King and Courts, 1989; NG et al., 1990; Wheeler and Stroup, 1993). Subsequent studies investigated the cellular response in root resorption process (Lara et al., 2003) and the presence of serum IgG and IgM levels was shown in patients with apical root resorption resulting in dental trauma (Hidalgo et al., 2005). And more recently, Ramos et al. (2011), used saliva samples to determine the levels of secretory IgA anti-human dentin extract. Because there are few studies in literature about the antigenic components of the dentin in EARR, the present research, described here, aimed to partially characterize human dentin antigenic fractions detected through the saliva and additionally to evaluate the anti-dentin antibodies profile in patients undergoing orthodontic treatment. The subjects in this study had mild to severe root resorption and were evaluated during or post-treatment. This analysis could help in further studies to obtain early diagnosis, development of preventive

methods and understanding of the immunopathological mechanisms involved in root resorption.

METHODOLOGY

All procedures involving human were performed after consent given by the subjects or by a parent/legal guardian and were approved by the Human Ethics Committee at State University of Londrina.

Subjects' selection

This study had three different groups (A, B, C). The A study group had 13 subjects (mean age 16 ± 4 years old) with nine females and four males. This group was analyzed over two phases: 12 months following the beginning of the fixed appliance therapy, at which time the patients presented with radiographic signs of mild to moderate root resorption (T12); these subjects taken prior to treatment served as controls (T0). The B study group had 10 subjects (mean age 21 ± 2 years old) with five females and five males. This group was analyzed approximately 48 months following orthodontic treatment, at which time they presented with radiographic signs of mild to severe root resorption (PT). The C study group had ten volunteers matched by gender and age without treatment and no radiographic evidence of root resorption were used as controls (ST). Inclusion criteria were: no previous orthodontic treatment and no radiographic evidence of root resorption. Exclusion criteria were: trauma to the primary or permanent dentition, autoimmune disease, chronic inflammatory disease, periodontal disease, periapical lesions, asthma, active caries or oral mucosa lesions, use steroidal or non-steroidal anti-inflammatory drugs for at least one month before sampling occurred.

Saliva samples

Saliva samples were collected at T0, T12, ST and PT. These samples were obtained between 10:00 and 16:00 to avoid the effect of cardiac cycle in salivary IgG secretion into saliva. Unstimulated whole saliva samples (2 ml) were collected by expectoration into sterilized vials after the subjects had rinsed their mouth twice with water. Saliva samples were centrifuged at 12000 rpm for 10 min, and the supernatants were then stored at -20°C until use.

Antigen preparation

Extract of human dentin containing NCP from the dentin matrix was used as the antigen. Powder dentin was obtained using a modified technique described by Wheeler and Stroup (1993); the third molars were donated by patients for whom extraction was indicated. The dentin was removed using a drill with a high-speed bit. The powder obtained was diluted at demineralizing solution (guanidine-HCl 5 M, 10%, ethylenediamine tetraacetic acid pH 5.0 and $1 \mu\text{M}$ phenylmethylsulfonyl fluoride) for 14 days at 4°C and then centrifuged two times at 3.218 rpm for 20 min. The supernatant was collected. The protein content was assessed by the Lowry method (1951). Dentin extract was stored at -80°C until use.

Radiographs

Periapical radiographs were obtained from subjects at T0, T12, ST and PT (70 kV, 10 mA, exposure time 0.7 s). The radiographs of the upper central incisors were taken by using the long cone paralleling technique. Kappa values for the intra-examiner variation ranged from 0.85 to 0.9. The most resorbed incisor was considered

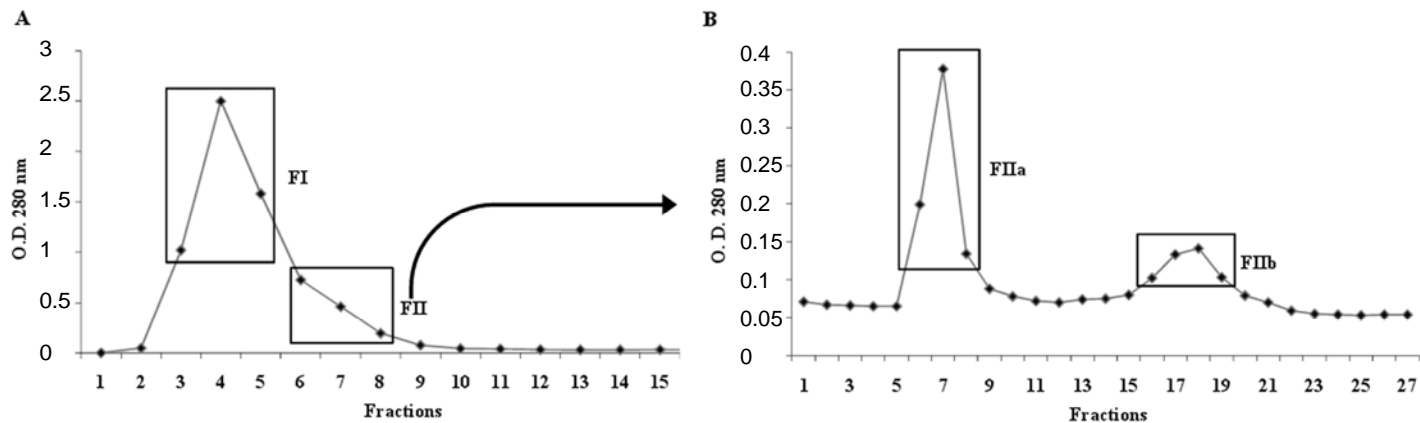


Figure 1. Chart of Gel filtration chromatography - Human dentin extract. (A) Fraction FI (tubes 3, 4 and 5) and fraction FII (tubes 6, 7 and 8) were fractionated in a Sephadex G-25 column. (B) Fraction FII was refractionated in a Sephadex G-100 column, yielding fraction FIIa (tubes 6, 7 and 8) and fraction FIIb (tubes 16, 17, 18 and 19). Only fractions FI and FIIa were used in these experiments.

for analysis. Tooth length was measured from the incisal edge to the apex. The measurements were made with a pachymeter (0.02 mm precision; Mitutoyo Sul Americana, São Paulo, Brasil) placed parallel to the pulp canal. Image distortion was determined by comparing the image length of a radiopaque object (metal clips) placed on the film. Image distortion between T0 and T12 radiographs was determined by comparing crown length. The maximum acceptable distortion was 5%. For analysis of degree of resorption, upper central incisor was selected, in subjects after treatment with orthodontic appliances. The degree of root resorption was classified by using criteria described by Levander and Malmgren (1988): mild resorption or degree I- with irregular apical root contour; moderate resorption or degree II- lesser 2 mm, severe resorption or degree III- 2 mm to one-third of the original root length. The patients presented Class I or Class II malocclusion.

Gel filtration chromatography on Sephadex G-25 and Sephadex G-100

To desalinate the extract, crude dentin extract was fractionated in a Sephadex G-25 gel filtration column (Sigma, St. Louis, USA). The automatic fraction collector (FC 203B; Gilson, Middleton, USA) was maintained at 4°C. Only one fraction was concentrated and refractionated on a Sephadex G-100 column (Sigma, St. Louis, USA). The eluted fractions in phosphate buffered saline (PBS) were read in a spectrophotometer at 280 nm (Pharmacia Biotech, Sweden). The total protein content of each fraction was assessed by the Lowry method (1951).

ELISA for the detection of anti-dentin salivary IgG

ELISA immunoplates (Techno Plastic Products, Zurich, Switzerland) were sensitized with 30 µg/mL FI or FIIa fractions in carbonate-bicarbonate buffer (Na₂CO₃ 1.59 g, NaHCO₃ 2.93 g, distilled water, qsp 1000 ml, pH 9.6), incubated for 1 h at 37°C and overnight at 4°C. The plate was washed four times with phosphate-buffered saline pH 7.2 (PBS), containing 0.05% Tween 20, and 0.5% skimmed milk. Then, the plates were blocked with PBS containing 0.5% Tween 20 and 5% skimmed milk for 1 h at room temperature. After washing, undiluted saliva samples were incubated at 37°C for 90 min. After washing, the conjugated goat-anti-human IgG-peroxidase (A8775; Sigma-Aldrich, St. Louis, USA) was added at 1:4000 dilution, and incubated for 90 min at 37°C. It

was added after washing the O-phenylenediamine substrate solution. The reaction was stopped after 15 min using 50 µL/well of 4NH₂SO₄. Absorbances were read at 492 nm in a Multiskan EX reader (Lab Systems, Helsinki, Finland). Antibody levels were expressed as absorbance in optical density units (O.D.).

SDS-PAGE electrophoresis and Western blot

FI and FIIa were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5-15% gradient acrylamide gels and were transferred to nitrocellulose membranes (NCM). The NCM was blocked with PBS containing 0.5% Tween 20 and 5% skimmed milk for 60 min h at room temperature. The NCM were cut into individual strips containing fractions F or FIIa and a molecular weight standard. Each strip was incubated with pools of undiluted saliva samples (T0, T12, ST or PT) with 10 samples from each group, followed by the addition of 1: 1000 conjugated goat-anti-human IgG-peroxidase (A8775; Sigma-Aldrich, St. Louis, USA) 90 min at 37°C and overnight at 4°C. After washing tetramethyl benzidine (00-2019; Invitrogen, San Francisco, USA) was added. The reaction was stopped with distilled water. The controls were saliva samples from the T0 and ST subjects.

Data analysis

The following non-parametric tests were used: the Wilcoxon test for paired groups (T0 and T12) and the Mann-Whitney test for unpaired group (ST and PT). These tests were used to detect differences in the ELISA absorbances (antibody levels expressed as O.D.). The results were considered statistically significant when P<0.05. The Lilliefors and Shapiro-Wilks normality tests were used.

RESULTS

Gel filtration chromatography

Chromatography of dentin extract on a Sephadex G-25 column resulted in one main peak of light absorption at 280 nm. From this peak, two fractions were obtained: FI and FII (chart demonstrated in Figure 1A). The FI

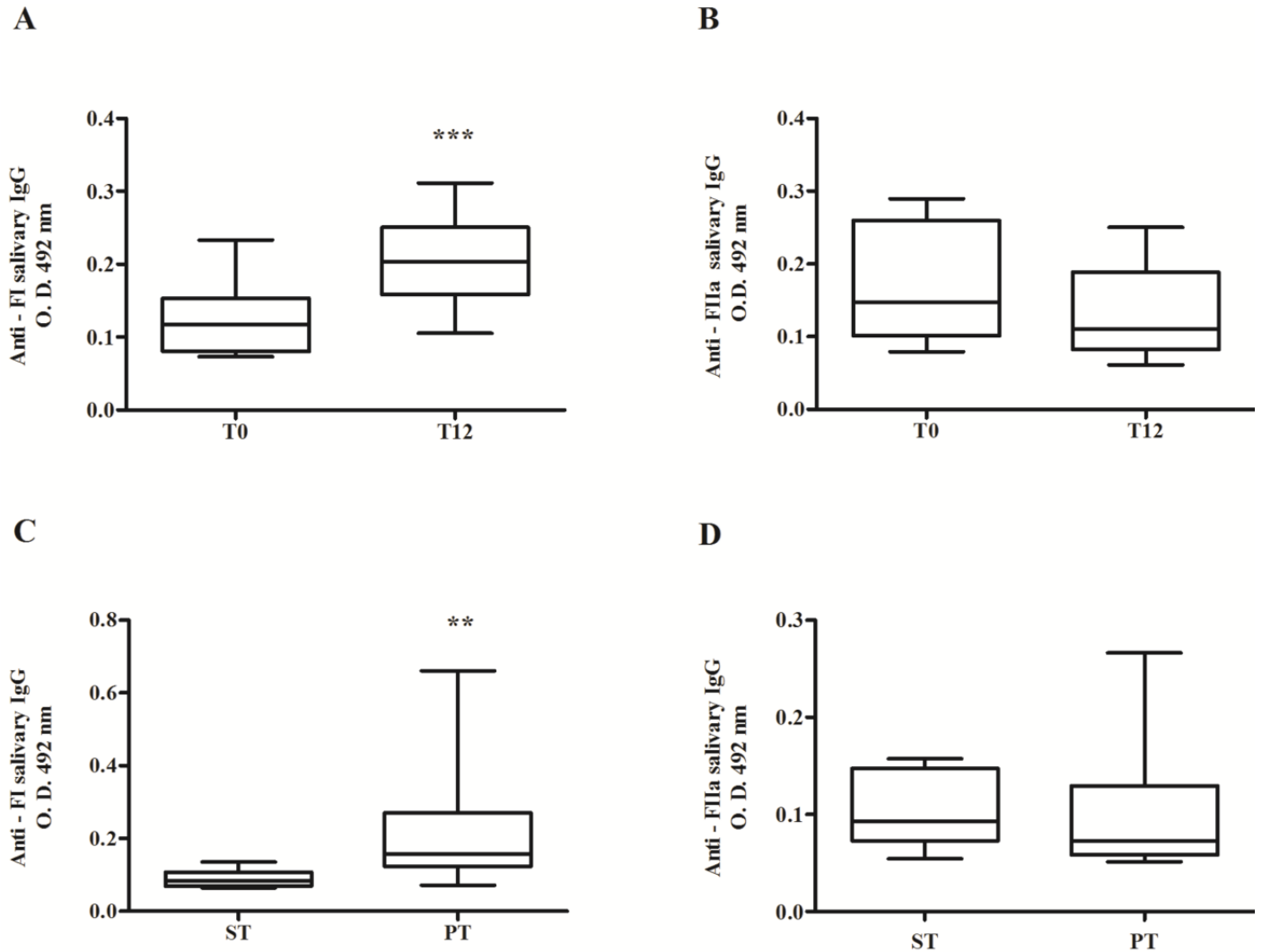


Figure 2. Analysis of the levels of salivary IgG against FI or FIIa fractions of human dentin extract using ELISA assays. Statistically significant differences ($P < 0.05$) are marked with asterisks. (A) Salivary IgG against FI fraction of human dentin extract in T0 or T12. (B) Salivary IgG against FIIa fraction of human dentin extract in T0 or T12. (C) Salivary IgG against FI fraction of human dentin extract in patients PT or ST. (D) Salivary IgG against FIIa fraction of human dentin extract in patients in PT or ST.

fraction was concentrated by lyophilization and was refractionated on a Sephadex G-100 column, resulting in a 280 nm spectrophotometric profile containing two peaks. Fractions corresponding to each peak were mixed, resulting in pool FIIa and FIIb (chart demonstrated in Figure 1B). Only fractions FI and FIIa were used in the following experiments.

Anti-FI and anti-FIIa salivary IgG levels detected by ELISA pre-treatment (T0) and after 12 months of orthodontic treatment (T12)

The anti-FI salivary IgG levels was higher at T12 than at T0 ($P < 0.05$, Wilcoxon test), as is shown in Figure 2A. No statistically significant differences were observed in anti-

FIIa salivary IgG levels between T0 and T12 ($P > 0.05$, Wilcoxon test), as is demonstrated in Figure 2B.

Anti-FI and anti-FIIa salivary IgG levels detected by ELISA in control donors (ST) and in patients approximately 48 months after orthodontic treatment (PT)

The anti-FI salivary IgG levels were higher in the PT group than in the ST group ($P < 0.05$, Mann-Whitney test; Figure 2C), but no statistically significant differences in the anti-FIIa IgG levels were observed between these groups ($P > 0.05$, Mann-Whitney test), as shown in Figure 2D.

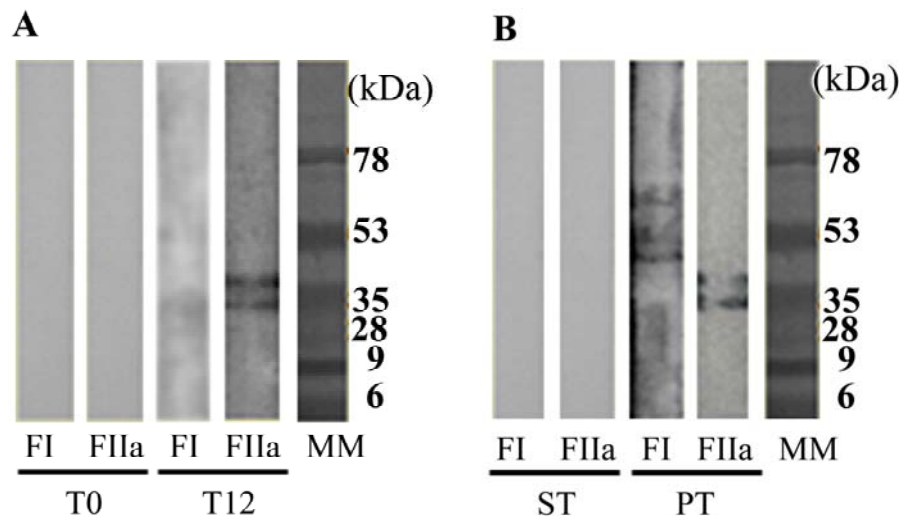


Figure 3. Western blot results of fractions FI or FIIa using pooled saliva samples from patients. (A) T0 or T12, (B) PT or ST.

FI and FIIa fraction analysis by Western blot

Western blot results show that the salivary IgG of the T12 or PT groups (but not the T0 or St groups) detected dentin antigens in the FI fraction (approximately 50 to 70kDa) and the FIIa fraction (approximately 35 kDa and 40 kDa). These data are shown in Figure 3A and 3B.

DISCUSSION

Based on the evidence that anti-dentin antibodies can be detected in animal experimental models and in patients with root resorption (King and Courts, 1989; Ng et al., 1990; Wheeler and Stroup, 1993; Hidalgo et al., 2005; Ramos et al., 2011), it has been hypothesized that pathological root resorption may be associated with autoimmune responses against dentinal components. The development of autoimmune disorders could be bound to the presence of autoreactive T lymphocytes in the thymus that have not been deleted (Weiner et al., 1995). An effective mechanism to prevent self-reactivity is the compartmentalization of certain self-antigen under normal conditions (Kindt et al., 2008). And the exposure of some hidden antigens, released after injury, makes them accessible to specific receptors of antigen-presenting cells (APCs) and adaptive immune cells, which can result in the production of autoantibodies (Janeway, 2007). The presence of anti-dentin autoantibodies in the saliva of control groups could be explained by the indexes of dental resorption described in the general population (Massler and Malone, 1954). However, the high level of specific autoantibodies observed in this study in T12 may be due to the release of dentinal antigens as consequence of orthodontic movement, compared to control, without treatment. When dentin antigen-IgG

complexes are internalized by APCs (macrophages, dendritic cells) and presented to autoreactive effector T cells, an inflammatory response will occur. Then, the antigen is eliminated, the ratio of IgG to antigen increases, decreasing immune responses and reducing tissue damage, with a slope of the response towards tolerance (De Groot et al., 2008). Natural regulatory T cell (nTreg) $CD4^+CD25_{Hi}FoxP3^+$, is an important mechanism of effector T cell regulation, and may represent one of the critical forms of autoregulatory response to self-antigens. On the other hand, the course of resorption will depend on a complex interaction of secreted molecules, inflammatory and bone cells within the tooth surrounding tissues (Ne et al., 1999).

The results shown, also indicated a significantly higher level of anti-FI fraction salivary IgG in (PT) than subjects (ST). This fact could be due to the persistence of the stimulus and the high half-life of the memory cells that produce antibodies anti-dentinal components (Schitteck and Rajewsky, 1990; Slifka, 1998). Thus, the induction of inflammatory events by dentin molecules released into microenvironment may contribute to the maintenance of this process (Silva et al., 2004). Western blot analysis of the FI fraction also detected another high molecular weight band that occurred in all the samples, including the control groups (data not shown). No significant difference in the FIIa fraction was observed between the groups and its control using an ELISA. However, western blot analysis detected dentinal antigens of approximately 35 and 40 kDa by saliva of the T12 and PT groups but not in the control groups. The concentration of FIIa fraction by lyophilization was necessary to detection in western blot, by salivary IgG. The results often contradictory in the literature about *in vivo* and *in vitro* dentin studies can be explained by the use of different extraction methods, source of dentin and methods of cell

treatment. Based on data of this study, we hypothesized that not all dentinal proteins may be immunogenic and some of them may be tolerogenic. It is possible that in the case of EARR, specially, mild or moderate, anti-dentin natural autoantibodies have a homeostatic function in mediating removal of dentinal components released within periodontal ligament microenvironment (HANS et al., 2007). In conclusion, our results suggest that antigenic fractions of approximately 35 to 70 kDa may be released from dentin, presented to the immune system and detected by salivary IgG in orthodontic patients with mild to severe root resorption. The high levels of salivary IgG to fraction of human dentin following orthodontic treatment may remain elevated even years after completion of therapy. The use of saliva samples provide great advantages of adherence by patients and may be a route of choice for the development of preventive methods, early diagnosis and understanding of the immunopathological mechanisms involved in the root resorption process. Further studies, with a larger number of patients, should be performed to confirm these results.

Conflict of Interests

The author(s) have not declared any conflict of interests

REFERENCES

- Al-Qawasmi RA, Hartsfield JK Jr, Everett ET, Flury L, Liu L, Foroud TM, Macri JV, Roberts WE (2003). Genetic predisposition to external apical root resorption in orthodontic patients: linkage of chromosome-18 marker. *J. Dent. Res.* 82:356-360.
- Apajalahti S, Peltola J (2007). Apical root resorption after orthodontic treatment-a retrospective Study. *Eur. J. Orthod.* 29: 408-412.
- Artun J, Van't Hullenaar R, Doppel D, Kuijpers-Jagtman AM (2009). Identification of orthodontic patients at risk of severe apical root resorption. *Am. J. Orthod. Dent. Orthop.* 135:448-455.
- Bassaran G, Ozer T, Kaya FA, Hamamci O (2006). Interleukins 2, 6, and 8 levels in human gingival sulcus during orthodontic treatment. *Am. J. Orthod. Dent. Orthop.* 130:7e1-7e6.
- Brin I, Tulloch JF, Koroluk L, Philips C (2003). External apical root resorption in Class II malocclusion: a retrospective review of 1-versus 2-phase treatment. *Am. J. Orthod. Dent. Orthop.* 124:151-156.
- Butler WT (1998). Dentin matrix proteins. *Eur. J. Oral Sci.* 106: 204-210.
- Consolaro A (2005). Reabsorções dentárias. Dental Press Maringá.
- De Groot AS, Moise L, McMurry JA, Wambre E, Van Overtvelt L, Moingeon P, Scott DW, Martin WX (2008). Activation of natural regulatory T cells by IgG Fc-derived peptide "Tregitopes." *Bl.* 8: 3303-3311.
- Dudic A, Giannopoulou C, Martinez M, Montet X, Kiliaridis S (2008). Diagnostic accuracy of digitized periapical radiographs validated against micro-computed tomography scanning in evaluating orthodontically induced apical root resorption. *Eur. J. Oral Sci.* 116:467-472.
- Hans UL (2007). Homeostatic roles of naturally occurring antibodies: An overview. *J. Autoimm.* 29: 287-294.
- Hidalgo MM, Itano EN, Consolaro A (2005). Humoral immune response of patients with dental trauma and consequent replacement resorption. *Dent. Traumatol.* 21:218-221.
- Janeway CA, Travers P, Walport M (2007). *Imunobiologia*. Porto Alegre: Artemed.
- Kindt, TJ, Goldsby, RA, Osborne, BA (2008). Tolerância e Autoimunidade In: *Imunologia de Kuby*. Artemed. Porto Alegre.
- King G, Courts F (1989). Humoral immune response to active root resorption. In: Norton LA, Burstone CJ, editors. *The Biology of Tooth*. Boca Raton: Movement CRC 276-285.
- Lara VS, Figueiredo F, da Silva TA, Cunha FQ (2003). Dentin-induced *in vivo* inflammatory response and *in vitro* activation of murine macrophages. *J. Dent. Res.* 6:460-465.
- Levander E, Malmgren O (1988). Evaluation of the risk of root resorption during orthodontic treatment: a study of upper incisors. *Eur. J. Orthod.* 10:30-38.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Marques LS, Ramos-Jorge ML, Rey AC, Armond MC, Ruellas AC (2010). Severe root resorption in orthodontic patients treated with the edgewise method: prevalence and predictive factors. *Am. J. Orthod. Dent. Orthop.* 384-388.
- Massler M, Malone AJ (1954). Root resorption in human permanent teeth. *Am. J. Orthod.* 40: 619-633.
- Nakagawa M, Kaneda T, Morita S, Sato T, Yomada T, Hanada K, Kumegawa M, Hakeda Y (2000). Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts. *FEBS. Let.* 473:161-164.
- Ne RF, Whitterspoon DE, Gutmann JL (1999). Tooth resorption. *Quint.* 30:9-25. Nesbitt SA, Horton
- MA (1997). Trafficking of matrix collagens through bone-resorbing osteoclasts. *Sci.* 276:266-269.
- Ng KT, King GJ, Courts FJ (1990). Humoral immune response to active root resorption with a murine model. *Am. J. Orthod. Dent. Orthop.* 98:456-462.
- Ogata Y, Niisato N, Moriwaki K, Yokota Y, Furuyama S, Sugiyama H (1997). Cementum, root dentin and bone extracts stimulate chemotactic behavior in cells from periodontal tissue. *Comp. Biochem. Physiol.* 116: 359-365.
- Ramos SP, Ortolan GO, Dos Santos LM, Tobouti PL, Hidalgo MM, Consolaro A, Itano EN (2011). Anti-dentine antibodies with root resorption during orthodontic treatment. *Eur. J. Orthod.* 33: 584-591.
- Sameshima GT, Sinclair PM (2001). Predicting and preventing root resorption: Part II Treatment factors. *Am. J. Orthod. Dent. Orthop.* 119:511-515.
- Schittek B and Rajewsky K (1990). Maintenance of B-cell memory by long-lived cells generated from proliferating precursors. *Nat.* 346:749-751.
- Segal GR, Schiffman PH, Tuncay OC (2004). Meta analysis of the treatment-related factors of external apical root resorption. *Orthod. Cran.* 7: 71-78.
- Silva TA, Rosa AL, Lara VS (2004). Dentin matrix proteins and soluble factors: intrinsic regulatory signals for healing and resorption of dental and periodontal tissues? *Oral Dis.* 10: 63-74.
- Slifka MK, Antia R, Whitmire JK, Ahmed R (1998). Humoral immunity due to long-lived plasma cells. *Imm.* 8:363-372.
- Wheeler TT, Stroup SE (1993). Traumatic root resorption in dentine-immunized mice. *Am. J. Orthod. Dent. Orthop.* 103: 352- 357.
- Wheiner HL, Miller A, Khoury SJ et al. (1995). In: J. Mestecky et al. *Advances in Mucosal Immunology*. Plenum Press New York 1243-1244.