The Effect of Three Different Rotary Instrumentation Systems on Substance P and Calcitonin Gene-related Peptide Expression in Human Periodontal Ligament

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Abstract

Introduction: The purpose of this study was to quantify the effect of three different rotary root canal preparation systems on substance P and calcitonin gene-related peptide expression in healthy human periodontal ligament. **Methods:** Fifty periodontal ligament samples were obtained from healthy premolars in which extraction was indicated for orthodontic reasons. Before extraction, 40 of these premolars were equally divided into four groups, and root canals were prepared using four different systems: the ProTaper Universal rotary system, the RaCe rotary system, the Mtwo rotary system, and the hand instrumentation technique. The remaining 10 healthy premolars that were extracted without treatment served as a negative control group. All periodontal ligament samples were processed, and SP and CGRP were measured by radioimmunoassay. **Results:** Greater SP and CGRP expression were found in the ProTaper Universal group followed by the hand instrumentation group, the RaCe, and the Mtwo groups. The lower SP and CGRP values were for the negative control group. The Kruskal-Wallis test showed statistically significant differences between groups ($p < 0.0001$). Post hoc Least Significant Difference (LSD) tests showed statistically significant differences in SP and CGRP expression between the negative control group and all the other groups except the Mtwo group. Hand instrumentation also showed statistically significant differences with all the other groups, except the ProTaper Universal group. Differences between the three rotary systems were also statistically significant. **Conclusion:** SP and CGRP expression in periodontal ligament increases when teeth are prepared with ProTaper Universal and RaCe rotary instrumentation systems as well as with hand instrumentation. Mtwo maintains SP and CGRP levels. *(J Endod 2010;36:1938–1942)*

Key Words

Calcitonin gene-related peptide, human periodontal ligament, neurogenic inflammation, rotary instrumentation, substance P

A frequent problem in endodontics is the development of post-treatment symptomatic apical periodontitis, which may vary from a low-intensity sensitivity when biting over the tooth to a severe pain to even the slightest touch (1). Apical periodontitis is defined as a circumscribed inflammation of the periodontal ligament in the apical region and it has been reported to be provoked by extrusion of different irritants from the root canal system (such as dentin debris, necrotic tissue, microorganisms, irrigants and/or filling materials) towards the periapex during canal preparation, generating an antigen-antibody reaction with the correspondent inflammatory reaction, even when working length is well established (2, 3).

Similar to dental pulp, periodontal ligament inflammation has a neurogenic source, which is induced by the release of neuropeptides from periapical tissue C-type nerve fibers, after being injured during root canal therapy (4). Substance P (SP) and calcitonin gene-related peptide (CGRP) are capable of triggering vasodilation, plasma extravasation, immune system activation, chemotaxis, recruitment, and/or regulation of inflammatory cells such as macrophages, mast cells, and lymphocytes (5). Finally, the release of inflammatory mediators in the periodontal ligament generates vascular stasis in the affected area (6, 7). Recent evidence has suggested that human fibroblasts are able to produce SP and that neuropeptides could also regulate the expression of angiogenic growth factors in fibroblasts, suggesting that these cells also play a role in neurogenic inflammation (8, 9). These biological effects could explain the clinical events of pain and inflammation observed during symptomatic apical periodontitis after root canal therapy (10).

It has been reported that the severity of periodontal ligament inflammation is directly proportional to the degree of the tissue damage (ie, the quantity of apically extruded debris [1, 11] and the mechanical stress exerted on the tooth [12]). It also has been shown that all root canal preparation techniques cause some degree of debris extrusion (11, 13–16). However, the amount of apically extruded irritants may vary according to the technique and the characteristics of the instrument used (3).

According to manufacturers, nickel-titanium rotary instruments, such as ProTaper Universal (Dentsply Maillefer, Ballaigues, Switzerland), Mtwo (VDW, Munich, Germany) and RaCe (FKG, La Chaux-de-Fonds, Switzerland), have been designed with different physical characteristics (ie, profile section, core diameter, rake angle,

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0099-2399/$ - see front matter

doi:10.1016/j.joen.2010.08.043

variable helicoidal angle, variable distance between flutes, or pitch) in order to reduce the quantity of debris extruded into the periodontal ligament. It has been stated that the profile section of an instrument establish the size of its core and together with the distance between flutes are responsible of providing enough space to allow detritus to be removed coronally, therefore reducing apical extrusion (17). Moreover, instruments with a positive rake angle have more cutting effectiveness and suffer less torsional stress when working because they are not easily blocked, avoiding the instrument to pump debris into the periapical tissues and reducing the mechanical stress over the tooth (18). Taking into consideration that root canal preparation may trigger a neurogenic inflammation response in the periodontal ligament and that SP and CGRP play an important role during this inflammatory process, the purpose of this study was to quantify the effect of different root canal rotary instrumentation systems on SP and CGRP expression in healthy human periodontal ligament. This knowledge could be useful for assessing neuropeptide behavior when routine endodontic procedures are performed, and consequently, contribute to clinician's decision making to minimize tissue injury.

Materials and Methods

A descriptive comparative study was performed according to Colombian Ministry of Health recommendations regarding ethical issues in research involving human tissue. Written informed consent was obtained from each patient participating in the study (18-30 years old, healthy, not medicated, and nonsmoking human donors). Fifty periodontal ligament samples were obtained from 50 lower premolars in which extraction was indicated for orthodontic reasons. All teeth used were caries and restoration free with complete root development determined both visually and radiographically, without signs of periodontal disease or traumatic occlusion and without orthodontic forces. Teeth had only one straight canal (canal curvatures over 20° were not included).

Teeth were equally divided and randomly assigned into the following five groups: (1) ProTaper Universal, (2) RaCe, (3) Mtwo, (4) hand instrumentation, and (5) intact-teeth control group. All teeth were anesthetized by an inferior alveolar nerve block injection of 1.8 mL 4% prilocaine without vasoconstrictor. Adequate pulpal anesthesia was ascertained with a negative response to an electronic pulp vitality test.

Experimental Procedure and Sample Collection

For the intact-teeth control group, extraction was performed by conventional methods without excessive injury to the periodontal ligament 10 minutes after anesthetic application. For the rest of the groups, teeth were isolated with a rubber dam, cavity accesses were performed using a Zekrya bur (Dentsply, Tulsa, OK) in a high-speed handpiece, the working length was established with the aid of an apex locator (Root ZX II, J Morita, Japan) set to 0.5 mm and radiographically confirmed, and finally root canals were prepared with the corresponding preparation technique before extraction as follows: (1) the ProTaper Universal group: root canals were prepared using the Protaper Universal (Denstply Maillefer, Ballaigues, Switzerland) technique strictly following manufacturer's sequence and recommendations (ie, SX, S1, and S2 with brushing motion and F1, F2, F3, and F4 until reaching the working length without apical pressure), (2) the RaCe group: root canals were prepared using the RaCe (FKG, La Chaux-de-Fonds, Switzerland) technique strictly following manufacturer's sequence and recommendations (ie, PreRaCe 40/.10 and 35/.08 for preflaring and RaCe 20/.02, 25/.02, 30/.04, 35/.04 and 40/.04 reaching the working length with a brushing motion and without apical pressure), (3) the Mtwo group: root canals were prepared using the Mtwo (VDW, Munich, Germany) technique strictly following manufacturer's sequence and recommendations (ie, 10/.04, 15/.05, 20/.06, 25/.06, 30/.05, 35/.04, and 40/.04, all of them reaching working length without apical pressure), and (4) the hand instrumentation group: root canals were prepared with hand instrumentation using Flexofiles .02 taper (Denstply Maillefer, Ballaigues, Switzerland) from 10 to 40 to the working length with a filing motion.

Root canal preparations were performed by a single operator to avoid interoperator variation. All canal preparation techniques used in the study consist of seven files each. Files were used only one time and then they were discarded, and the preparation time did not exceed 10 minutes for each tooth. Apical patency was verified in all groups with a #10 K-file (Dentsply Maillefer, Ballaigues, Switzerland). Canals were irrigated with 1.5 mL of 5% sodium hypochlorite between each file with a Monojet syringe with a 30-G needle placed 3 mm short of the working length. Teeth were extracted 10 minutes later after ending canal preparation with conventional methods without excessive injury to periodontal ligament. After extraction, a #10 K-file was placed into the canal until its tip protrudes from the foramen to corroborate apical patency and that all working lengths were at 0.5 mm from the foramen. Periodontal ligament samples were obtained from the apical 3 mm of the root with a periodontal curette, placed on an Eppendorf tube, snap frozen in liquid nitrogen, and kept at −70°C until use.

Radioimmunoassay

Periodontal ligament samples were defrosted without thermal shock, dried on a filter, and weighed on an analytic balance. Neuropeptides were extracted by adding 150 μL of 0.5 mol/L of acetic acid and double boiling in a thermostat bath for 30 minutes in accordance with previously reported protocols (19–22). SP and CGRP expression were determined by competition binding assays using human SP and human CGRP-radioimmunoassay (RIA) kits (RK-061-05 and RK-015-02; Phoenix Peptide Pharmaceutical, Belmont, CA). Two different RIA assays were conducted, one for each neuropeptide.

In both RIA assays, a total of 50 μL of each sample solution was incubated in polypropylene tubes at room temperature for 20 hours with 100 μL of primary antibody (for each neuropeptide) and 100 μL of different SP (or CGRP) concentrations (10 pg/mL-1,280 pg/mL). Then, 50 μL of 125I-SP (or 125I-CGRP) was added and left to incubate for another 24 hours. Bound fractions were precipitated by the addition of 100 μL of a secondary antibody (goat antirabbit immunoglobulin G serum), 100 μL of normal rabbit serum, and 500 μL of RIA buffer containing 1% polyethylene glycol 4000. After 2 hours of incubation at room temperature, tubes were spun at 3,000 rpm for 45 minutes at 4°C. The supernatants were decanted, and pellet radioactivity was read on a Gamma Counter (Gamma Assay LS 5500; Beckman, Fullerton, CA). Standard curves of authentic peptide were made in buffers identical to the tissue extracts on semilog graph paper.

Finally, analysis of the binding data assessed the amount of SP and CGRP present in every sample using the percentage of maximum binding (B/B0%) calculated for each unknown sample and reading across the graph to the point of intersection with the calibration curve where the corresponding x-axis coordinate is equivalent to the concentration of peptide in the assayed sample.

Statistical Analysis

Values are presented as SP and CGRP concentrations in picomoles per milligram of periodontal ligament. The mean standard deviation and maximum/minimum values are presented for each group. The Kruskal-Wallis test was performed to establish statistically significant

TABLE 1. SP Expression in the Periodontal Ligament from Healthy Human Premolars after the Root Canal Preparation with Different Systems

	N	Mean*	Standard Deviation	Minimum	Maximum
Intact teeth†	10	0.430	0.049	0.350	0.500
Hand Instrumentation‡	10	1.194	0.142	1.010	1.470
ProTaper Universal	10	1.219	0.107	1.020	1.380
RaCe	10	0.869	0.098	0.670	0.990
Mtwo	10	0.512	0.115	0.380	0.730

SP, substance P.

*Values are presented as the SP concentration in picomoles per milligram of the periodontal ligament. The Kruskal-Wallis test showed statistically significant differences between groups ($p < 0.0001$).

†The LSD post hoc test showed significant differences with all the other groups except with the Mtwo group.

‡The LSD post hoc test showed significant differences with all the other groups except with the ProTaper Universal group.

differences between groups ($p < 0.05$). LSD post hoc comparisons were also performed.

Results

Both neuropeptides were found to be present in all periodontal ligament samples (Tables 1 and 2). The highest SP levels were observed in the ProTaper Universal group, with a mean value of 1.219 ± 0.107 pmol of SP per mg of periodontal ligament followed by the hand instrumentation group with a mean SP value of 1.194 ± 0.142 pmol of SP per mg of periodontal ligament. The mean value for the RaCe group was 0.869 ± 0.098 pmol of SP per mg of periodontal ligament. The lowest SP levels were observed in the Mtwo group and in the intact-teeth control group samples with a mean value of 0.512 ± 0.115 and 0.430 ± 0.049 pmol of SP per mg of periodontal ligament, respectively (Fig. 1). The Kruskal-Wallis test showed statistically significant differences between groups ($p < 0.0001$). LSD post hoc tests showed significant statistical differences between the intact-teeth control group and the ProTaper Universal, RaCe, and Hand instrumentation groups ($p < 0.001$). Differences between the Mtwo group and the other experimental groups were also statistically significant ($p < 0.0001$). There was no statistically significant difference between the intact-teeth and the Mtwo groups ($p = 0.08$). Differences between hand instrumentation and Protaper Universal groups were not statistically significant also ($p = 0.57$).

The highest CGRP levels were observed in the ProTaper Universal group, with a mean value of 0.0832 ± 0.008 pmol of CGRP per mg of periodontal ligament followed by the hand instrumentation group with a mean value of 0.0785 ± 0.009 pmol of CGRP per mg of periodontal ligament. The mean value for the RaCe group was 0.0438 ± 0.012 pmol of CGRP per mg of periodontal ligament. The lowest CGRP levels were observed in the Mtwo group and in the intact-teeth control group samples with a mean value of 0.0250 ± 0.017 and 0.0180 ± 0.0068 pmol of CGRP per mg of periodontal ligament, respectively (Fig. 2). The Kruskal-Wallis test showed statistically significant differences between groups ($p < 0.0001$). LSD post hoc tests showed significant statistical differences between the intact-teeth control group and the ProTaper Universal, RaCe, and Hand instrumentation groups ($p < 0.001$). Differences between the Mtwo group and the other experimental groups were also statistically significant ($p < 0.001$). There was no statistically significant difference between the intact-teeth and the Mtwo groups ($p = 0.13$). Differences between hand instrumentation and Protaper Universal groups were not statistically significant ($p = 0.17$).

Discussion

The present study is novel as is the first one that tried to establish the amount of neuropeptides (SP and CGRP) released in human periodontal ligament as a result of root canal preparation in order to find a biological answer to one common problem in clinical endodontics known as symptomatic apical periodontitis. However, it is important to point out that periodontal ligament inflammation is multifactorial; and it may be influenced by several features of root canal preparation, such as mechanical stress, debris and/or irrigant extrusion, and apical patency verification (12–16).

SP and CGRP values were obtained from healthy premolars in which extraction was indicated for orthodontic reasons. The local anesthetic used in all groups of this study was 4% prilocaine without vasoconstrictor to prevent neuropeptide expression from becoming attenuated by vasoconstrictors as previously shown (21, 23). In order to minimize the variables that could lead to differences between one system and another, the whole root canal preparation procedures were standardized. A total of seven instruments were used for each group: the main apical file for all groups was a 0.40-mm tip diameter and preparation time did not exceed 10 minutes. The irrigation protocol was also standardized because it has been shown that irrigant used during root canal preparation can also be extruded causing chemical irritation that directly affects the periodontal ligament (24).

There was a 10-minute delay after completing the root canal preparation technique before proceeding with tooth extraction. The extraction procedure was also standardized to affect all teeth equally; it was performed in less than 5 minutes and without excessive injury to periodontal ligament. Because SP release is immediate, calcium dependent,

TABLE 2. CGRP Expression in the Periodontal Ligament from Healthy Human Premolars after Root Canal Preparation with Different Systems

	N	Mean*	Standard Deviation	Minimum	Maximum
Intact teeth†	10	0.0180	0.0068	0.0111	0.0307
Hand Instrumentation‡	10	0.0785	0.0090	0.0689	0.0942
ProTaper Universal	10	0.0832	0.0082	0.0706	0.0984
RaCe	10	0.0438	0.0123	0.0312	0.0692
Mtwo	10	0.0250	0.0174	0.0121	0.0714

CGRP, calcitonin gene-related peptide.

*Values are presented as CGRP concentration in picomoles per milligram of the periodontal ligament. The Kruskal-Wallis test showed statistically significant differences between groups ($p < 0.0001$).

†The LSD post hoc test showed significant differences with all the other groups except with the Mtwo group.

‡The LSD post hoc test showed significant differences with all the other groups except with the ProTaper Universal group.

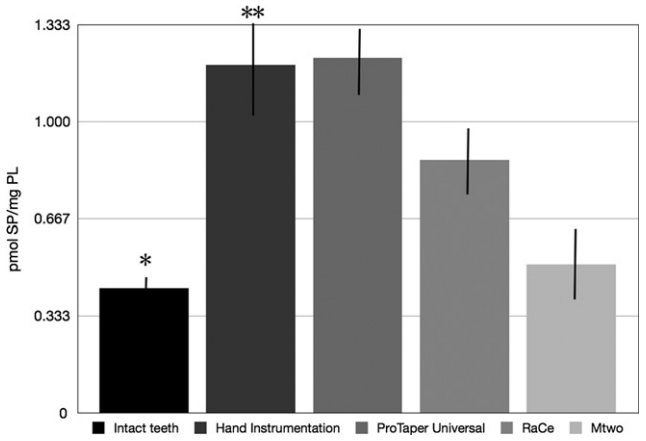

Figure 1. SP expression in periodontal ligament from healthy human premolars after the root canal preparation with different systems. *Statistically significant differences with all other groups except the Mtwo group. **Statistically significant differences with all other groups except the ProTaper Universal group.

and of short-term, this period of time appears to be sufficient for allowing the neuropeptide to be released from terminal fibers before being degraded by endogenous peptidases (25, 26). Other authors (27, 28) have speculated on the possible mechanisms for the increase of extracellular neuropeptides including (1) increased synthesis of the neuropeptide in the trigeminal ganglia; (2) an increased rate of transport; (3) an increased release; and (4) decreased levels of peptidases, which would result in decreased degradation of neuropeptides. More recent evidence has shown that messenger RNA transcripts are transported to peripheral terminals, suggesting that peptide synthesis could occur directly in the peripheral terminals (29). However, in the present study, it is assumed that increased SP levels are more related to increased release because 10 minutes would not be enough for the other hypothesis to take place.

Periodontal ligament samples were obtained only from the apical 3 mm of the root because it has been shown that root apex anatomy varies greatly in the last 3 mm, showing a high incidence of accessory foraminas besides the main apical foramen (30), which constitute portals of exit where debris extrusion could take place during root canal preparation and therefore irritating the surrounding tissue (31). It has been reported that hand preparation techniques produce a greater debris extrusion because of the filing movement in which the instrument acts like a piston, pumping debris and irrigant to the periapical area. Moreover, K-type files have a constant 40o helicoidal angle, giving it a lower ability for removing debris coronally and a greater tendency of accumulating it in the spaces between flutes (13, 14, 31). Therefore, it was expected that this group would generate a greater expression of both neuropeptides in comparison with the rotary systems group. Results of the present study are in accordance with the hypothesis stated, showing a significantly greater expression of SP and CGRP than the other groups, except for the ProTaper Universal.

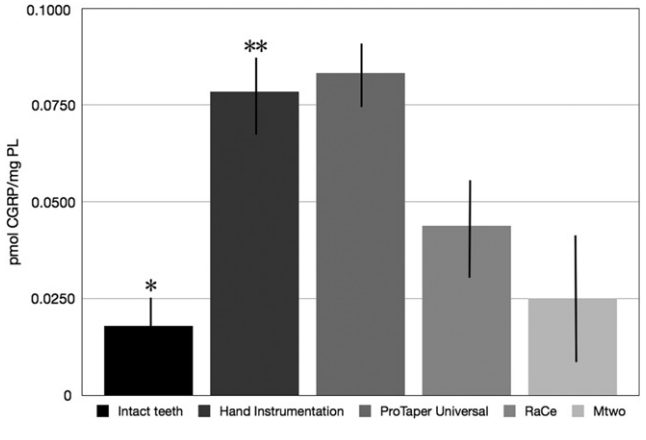

Figure 2. CGRP expression in periodontal ligament from healthy human premolars after the root canal preparation with different systems. *Statistically significant differences with all other groups except the Mtwo group. **Statistically significant differences with all other groups except the ProTaper Universal group.

ProTaper Universal, Mtwo, and RaCe rotary systems were selected for this study because they have certain design characteristics that, according to manufacturers, reduce debris apical extrusion, such as their variable helicoidal angle and variable pitch (17, 18, 32, 33). However, it is important to notice that the variable helicoidal angle and the variable pitch are similar between the three systems. Therefore, it could be inferred that these are not the most important design characteristics in reducing debris extrusion and that there may be other design characteristics that may be more determinant such as profile section, rake angle, and taper. These latter differences in the instrument's design could be responsible in the variability of neuropeptide expression in the present study.

Mtwo group showed a neuropeptide expression similar to the intact-teeth control group. This could be explained by the instrument S-shaped profile section, which gives the instrument a smaller core diameter, providing enough space to accumulate debris (17). Mtwo instruments also have a positive rake angle, which gives the instruments an efficient cutting action, and in conjunction with their variable helicoidal angle and pitch, the debris would tend to move coronally (32). On the other hand, the ProTaper Universal group showed the highest neuropeptides expression. This could be explained by the convex triangular profile section in the shaping files and in the finishing files 1 and 2, which provides greater mass to the instruments' core, making them more rigid, reducing the depth of flutes, and thereby limiting their ability to allow coronal removal of debris. The profile section in finishing files 3 and 4 was modified to a concave triangular profile section to make them more flexible. However, these concave areas do not provide sufficient space for the accumulation of debris, and the presence of variable tapers along the instrument facilitates the instrument to lock more easily and act like a piston promoting apical extrusion (33).

The RaCe group increased SP and CGRP expression significantly more than the Mtwo group but less than ProTaper Universal group. This could be explained by the design of the instruments, which had characteristics from both of the other two systems used in this study. RaCe instruments have straight cutting areas alternated with smaller twisted cutting areas. These alternated cutting areas have a positive rake angle, giving the instrument an efficient cutting action. Although it has a triangular profile section, its variable helicoidal angle and variable pitch provide enough space between flutes to allow the coronal remove of debris (34).

Results from the present study are in accordance with the hypothesis that neuropeptides contribute to the pathophysiology of peripheral inflammation and that root canal preparation generates an inflammatory process in the periapical tissues that could explain the post-treatment pain events after root canal therapy, such as symptomatic apical periodontitis (10, 35). It can be concluded that root canal preparation techniques increase SP and CGRP expression in human periodontal ligament, which could generate an inflammatory process in the periapical tissues. This neuropeptide increased release is

significantly higher when ProTaper Universal and RaCe rotary systems are used as well as hand instrumentation. The Mtwo group did not show statistically significant differences with the basal levels.

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