Research Article

Comparison of Inflammatory Markers in Saliva before and after Endodontic Treatment

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Abstract

In endodontic infections, inflammatory mediators such as cytokines are released, recruited and retained until the infection is eradicated. Root canal therapy is performed to prevent the spread of infection. The aim of this study was to investigate the effects of root canal debridement (cleaning and shaping) on periapical inflammation by measuring the levels of inflammatory cytokines, Interleukin-8 (IL-8) and Interleukin-10 (IL-10). The study includes twenty patients with pulp necrosis and asymptomatic apical periodontitis. Periradicular samples were collected using paper points before and after root canal debridement. Cytokine levels were determined by Sandwich Enzyme-Linked Immunosorbent Assay (ELISA). Data were analyzed using paired t-test (PASW Statistics 18) (P=0.05). All samples showed the presence of IL-8 and IL-10 prior to root canal debridement. Significantly reduced levels ($p<0.05$) of IL-8 and IL-10 were detected after root canal debridement. In conclusion, root canal debridement significantly decreased the levels of the tested pro- and anti-inflammatory cytokine in the periradicular interstitial fluid.

Keywords: Cytokines; Endodontic treatment; Inflammatory markers; IL-8; IL-10; Inflammatorymarkers; Saliva

Introduction

Root canal infections are induced by bacterial contamination of the pulp. [1,2] Following this, elevated amounts of inflammatory mediators are released by the innate and adaptive immune cells at the sites of infection.

Prolonged infection and inflammation leads to pulpal necrosis and development of periapical inflammation, eventually causing resorption of bone and periapical granuloma or cyst formation. [3]

In addition to the above-mentioned changes, lymphocytes are also found in abundance in the periapical inflammatory infiltrate. Periapical lesions are dominated by the presence of lymphocytes, followed by Polymorphonuclear Leukocytes (PMN), monocytes/macrophages and plasma cells and blasts.

Specific responses demonstrated by activation of lymphocytes and nonspecific responses such as (LPS) stimulated macrophages and PMN are responsible for the production of chemokines and cytokines, which not only help to combat root canal infection but also stimulate the alveolar bone resorption at the inflammatory site. [4–6] Cytokines, which are small signaling molecules released by the host immune cells, are known to be excellent markers of inflammation. Cytokines function by maintaining an equilibrium to prevent the constant state of inflammation and may be pro-inflammatory (e.g. Interleukin-8) or anti inflammatory (e.g. Interleukin-10). [7]

IL-8, also known as CXCL8, is an inflammatory chemokine which induces chemotaxis and activation of inflammatory cells. Furthermore, IL-8 is rapidly synthesised at the local sites of inflammation to recruit, activate and retain inflammatory cells at the site to combat infection continuously. IL-8 is heat and proteolysis resistant, and is able to resist an acidic environment, rendering it ideal to function at sites of inflammation, where it must withstand suboptimal environments. [7–9] Conversely, IL-10 serves as an important immunoregulatory cytokine with potent anti-inflammatory properties. IL-10 inhibits the expressions of pro-inflammatory cytokines and chemokines, thereby promoting healing at the sites of infection. [10] It has been proposed that IL-8 and IL-10 cytokines may be useful inflammatory markers, as they provide an overview of the

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immunological status of the pulp. Thus, the IL8//IL10 ratio has been recommended for further investigation and diagnosis of pulpal inflammation. An increase in the levels of IL-8 and IL-10 under inflammatory conditions in periapical and periodontal diseases has also been reported.

Microbial invasion from the root canal into the periapical tissues results in an imbalance between microbial factors and host immune response. Therefore, root canal therapy is performed to prevent or treat apical periodontitis, by eliminating infected tissue from the root canal space. Root canal treatment involves the use of instruments and chemical adjuncts to remove infected and inflamed tissue, as well as the microbial biomass from the root canal space. The healing of periapical tissues after root canal treatment appears to be regulated through the signalling of cellular matrix and molecular interactions which are promoted by an array of cytokines and other growth factors. [11] Besides root canal debridement, antiseptic dressing placed within the root canals as intracanal medicament helps in killing the microbes that remain within the root canal system after debridement. [12] Obtaining a microbe-free root canal system is important in creating a conducive environment to facilitate healing of the periapical tissues. [13]

The effects of root canal debridement procedures on the levels of pro- and anti-inflammatory cytokines remain unclear. It was the aim of this study to determine the effect of root canal debridement (cleaning and shaping) on the levels of IL-8 and IL-10 in periapical tissues *in vivo*. The null hypothesis was that root canal debridement does not significantly decrease the levels of IL-8 and IL-10.Previously our team has a rich experience in working on various research projects across multiple disciplines. [1–15] Now the growing trend in this area motivated us to pursue this project.

Materials and Methods

Patient selection twenty patients diagnosed with at least one tooth having pulp necrosis and asymptomatic apical periodontitis were included in this study, written informed consent was obtained from all the patients. Patients with acute symptoms, antibiotic consumption within 3 months prior to root canal therapy and retreatment cases were excluded. Pulp sensibility tests using cold and electric pulp testing were performed to diagnose the status of the pulp. Radiographs were obtained to confirm the presence of a single root with a single canal, and to confirm the periapical status.

Sample collection

Following administration of 0.6 mL of 2% lidocaine hydrochloride with epinephrine 1:80 000 the tooth to be treated was isolated using a rubber dam. Access cavity was prepared using size #2 and #4 round tungsten carbide burs. Once the canal orifices were reached, Gates Glidden burs were used to create straight line access. The pulp chamber was dried, and samples were collected by three paper points introduced consecutively into the canal to extend 2 mm beyond the apex (as measured radiographically) and left undisturbed for one minute. The paper points were then transferred to Thermo Scientific TM. Cryo-vial tubes and stored in a freezer at 80°C, until they were ready to be tested. Working length was then determined using an electronic apical foramen locator and confirmed radiographically. The root canals were then cleaned and shaped using stainless steel K-files, up to an apical size of 40. During root canal preparation, 10 mL of 3% sodium hypochlorite was used as the irrigant. The total duration of irrigation was 30 min. Once root canal preparation was completed, 2 mL of 17% EDTA was used for 1 min, and the root canals were rinsed with 2 mL of sterile normal saline. No intracanal medicament was placed inside the root canals and coronal accesses were restored with temporary material. After fourteen days, the teeth were re-accessed and samples were collected as mentioned previously. Following this, the root canals were then filled with gutta-percha and an epoxy resin root canal sealer using lateral compaction technique. Access cavities were then sealed with composite resin.

Cytokine analysis

The frozen specimens were thawed and, 250 lL of Assay Buffer A was added to each cryo-vial tube containing the samples. The vials were vortexed and centrifuged to allow complete mixing. This process was performed in triplicate. Levels of IL-8 and IL-10 were determined using the Biolegend LEGEND MAXTM Human IL-8 (CXCL8) ELISA Kit and LEGEND MAXTM Human IL-10 ELISA Kit, respectively, in accordance with the manufacturer's instructions. [15,16] The kit used was a Sandwich ELISA Kit consisting of a 96 wells strip plate which was pre-coated with capture antibody for IL-8, whereas the IL-10 kit was pre-coated with capture antibody for IL-10. The sensitivity for the capture antibodies were 4.4 and 2 pg mL1 for the IL-8 and IL-10 tests respectively.

Cytokine concentration

All reagents were brought to room temperature prior to usage. Top standard solution was prepared by diluting standard stock solution in Assay buffer A. Following this, sixtwo-fold serial dilution was performed with the prepared top standard solution in separate tubes using Assay Buffer A as a diluent. As a result, each tube consisted of 250, 125, 62.5, 31.3, 15.6 and 7.8 pg mL 1 of human IL-8 standard concentrations. Similarly, 62.5, 31.3, 15.6, 7.8, 3.9 and 1.95 pg mL1 of standard concentrations were prepared for IL-10. To measure the samples, 50 lL of Assay Buffer A was added to each well, followed by 50 lL of standard dilutions and samples were collected consecutively. The plate containing the wells was sealed and left to incubate overnight while shaking using the microplate shaker at 200 rpm under 4°C. After 24 h, the plates were washed four times, after which 100 lL of the detection antibody solution was added into the wells and incubated at room temperature while shaking on the microplate shaker. Later, the plates were re-washed four times, and 100 lL of Avidin Horseradish Peroxidase (AVIDIN-HRP A) solution was added and left to incubate for 30 min, while shaking, after which the washing procedure was repeated for another five times. Then, 100 lL of Substrate Solution F was added in the dark and incubated for

15 min. Finally, stop solution was added and data were interpreted at 450 and 570 nm using a spectrophotometer.

Based on the readings obtained from the spectrophotometer, the cytokine concentration levels of IL-8 were determined and expressed in picograms/millilitre of total protein. These steps were repeated to determine IL-10 levels.

Data analysis

Readings obtained from the spectrophotometer were used to plot a straight-line graph.

Based on the plotted graph, the concentrations of each standard dilutions and individual samples were then determined.

Paired t-test was used to calculate the statistical differences between the two groups (before and after) using SPSS PASW® Statistics 18 software.

Results

At baseline analysis, the presence of IL-8 levels was detected in 100% of the clinical samples (20 out of 20) with values ranging from 0.12 to 1112.46 pgm L1.

There was a significant difference in the IL-8 levels after root canal debridement in 15 patients ($P<0.05$), whereas five patients demonstrated an increase in IL-8 levels although the levels were not statistically significant [Figure 1].

The presence of IL-10 was also detected at baseline analysis in all the clinical samples that were collected (20 out of 20) with values ranging from 0.39491 to 53.6936 pgmL1.

There was a significant decrease in IL-10 levels after root canal debridement in 16 patients ($P<0.05$) whereas the remaining four patients had demonstrated an increase in IL-10 levels; however, the levels were not statistically significant [Figure 2].

During data analysis, four samples were excluded.

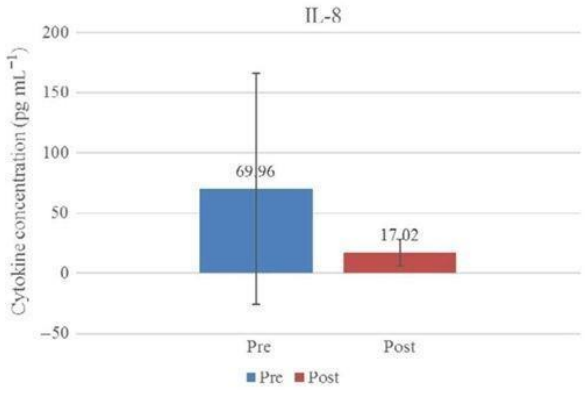

Figure 1: IL-8 levels in pre and post root canal debridement.

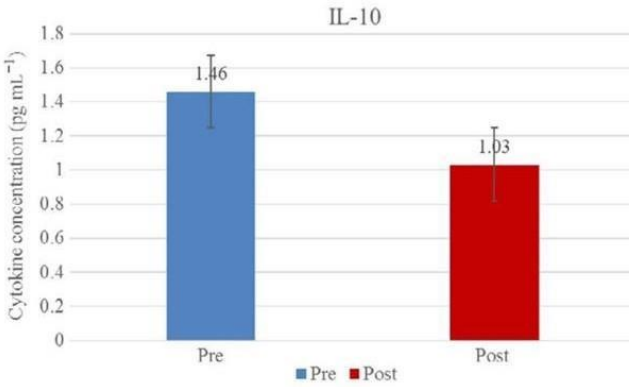

Figure 2: IL-10 levels in pre and post root canal debridement.

Discussion

The overwhelming response of microbial invasion of the root canal system against the body's defences is largely responsible for periapicalpathoses. [6] Thus, the inflammatory process initiates a host response in an attempt to eliminate the microorganisms and to prevent their spread into the adjacent tissue spaces. Several classes of body cells such as PMNs, lymphocytes, osteoclasts and epithelial cells are signalled and assembled to take part in this process. Simultaneously, the cytokines are released as part of the progression of the pathology. [6,17] Our institution is passionate about high quality evidence based research and has excelled in various fields. [16–22]

This study showed that the cytokine IL-8 was present in all clinical samples with pulpal necrosis (immaterial of the periapical status). It has been demonstrated previously that caries-exposed pulps show significantly higher levels of IL-8 compared to normal teeth. The presence of increased levels of IL-8 in inflamed pulps, compared to normal pulps has also been reported previously. IL-8 levels were also found to be increased among patients with severe odontogenic infections, while immunoregulators were suppressed during this period.

Interestingly, IL-8 has been suggested as a biomarker for observing sepsis, infections, and mortality in burn patients. Although IL-8 has been reported to be produced at early stages of inflammation, one of the unique abilities that IL-8 possesses in comparison to other cytokines is the longevity of IL-8 to retain its levels at the inflammatory sites for days or even weeks to combat the infection continuously. Similarly, the results of the present study demonstrated detectable levels of IL-10 in the periapical interstitial fluid prior to root canal cleaning and shaping. Hence, it can be inferred that anti-inflammatory cytokines are present in the inflammatory environment as described in other studies. [22,23]

In the current study, cytokine IL-8 levels were significantly decreased after cleaning and shaping (<0.05). Bambirra et al. reported that MRNA expression of IL-8 and other pro-inflammatory cytokines decreased 7 days after chemo-mechanical debridement, due to the reduction in microbial load. Furthermore, intracanal medication placement has also decreased the expression of Th1 type cytokines which are pro-

inflammatory in nature. [24] Sodium hypochlorite as a potent antimicrobial agent [25] is believed to have contributed to the above finding. From this study, it is evident that cleaning and shaping procedures alone has caused a significant reduction in recruitment and de-activation of IL-8 and IL-10 at the inflammatory sites.

Few studies [26,27] have used similar methodologies to assess the levels of inflammatory mediators and treatment outcomes after root canal treatment. Martinho et al. demonstrated that intracanal medicaments (chlorhexidine and calcium hydroxide) reduced bacterial counts and endotoxin levels. Furthermore, the levels of inflammatory cytokines IL-1b, TNF-a, and PGE2 were significantly decreased in the root canal. This established the positive correlations between bacterial counts and inflammatory cytokine levels. It was proposed that the decrease in inflammatory cytokine levels was attributable to both the anti-inflammatory and antimicrobial properties of the intracanal medicaments. From the perspective of healing outcomes, Duque et al. demonstrated that tooth mobility, probing depth and clinical attachment loss significantly reduced after root canal treatment and periodontal maintenance therapy. Consequently, it was suggested that the reduction of LPS and inflammatory cytokine levels following chemo-mechanical preparation lead to better prognosis of periodontal therapy. [27] Interestingly, correlations between interleukin levels and outcomes of treatment have also been established elsewhere, such as Temporomandibular Joint (TMJ) irrigation treatment wherein preoperative levels of IL-8 and IL-6 were associated with unsuccessful treatment outcome whereas preoperative levels of IL-10 was a significant predictor of successful outcome of treatment. [28] Taken collectively, it can be inferred that serum IL10, IL-12, IL-18 can be used as a predictor for treatment outcomes.

During data analysis, it was observed that two clinical samples had exceptionally higher levels of IL-8 when compared with the other samples. This finding might be associated with different bacteria strains present in the infected root canal. Gram-positive species have been shown to produce higher levels of IL-8 due to difference in activation of immunoregulatory systems against pathogens in the body. [29] Interestingly, it must also be noted that the highest IL-8 concentration levels detected in the pre-operative samples in this study was from a patient with Sjogren Syndrome. While it is known that patients with such autoimmune diseases have a dysregulated cytokine network which results in overexpression of local and systemic pro-inflammatory cytokines, [30] its implications on the findings of cytokine levels from root canals and periapical tissues have not been reported thus far. In addition to this, IL-8 levels in five out of 20 samples increased after cleaning and shaping. Such elevated levels may be associated with endodontic flare-ups. [31,32] Also, handling errors during sample collection and laboratory procedural errors might have led to the discrepancies. However, it remains uncertain whether the aforementioned issue's was/were due to endodontic flare up, microbial disturbances, human errors or a combination of all three.

Similarly, in case of IL-10, four samples were excluded during data analysis. This is because these samples had readings beyond the average concentration of the IL-10 cytokine and hence would interfere with the data analysis. The exact reasons for these outliers are unknown. It could be due to numerous factors, one being errors occurring during the collection of samples or testing of the samples involving laboratory procedures. Besides human error, heavy inflammation could also be the reason for the spurt in IL-10 levels. Host factors such as the patient's immune system could also be the reason for these extreme values. Interestingly, one of the patients was found to be suffering from secondary Sjogren syndrome. However, no strong evidence exists thus far, to support the correlation of the patient's immune system with the concentration of IL-10 found in periapical interstitial fluid. [33]

Although it has been reported that endodontic treatment of teeth with symptomatic or asymptomatic apical periodontitis has a success rate up to 86%-93% [34], endodontic failure is not uncommon. Higher chances of treatment failure have been associated with persistent bacterial contamination in the canal due to insufficient chemo-mechanical debridement. Other factors include missed canals, poor obturation quality, coronal leakage, over-instrumentation and procedural errors. [35] Since microbiological infections have been closely linked with host inflammatory response, the authors of this study suggest the development of chair side monitoring strategies for measuring levels of inflammatory biomarkers. Such chairside diagnostics could play an important role in preventing post-endodontic complications and reduce endodontic failure rates. [36] Furthermore, such methods will allow better understanding of progression and resolution of periapical disease secondary to root canal infections.

Conclusion

In conclusion, root canal debridement appears to be effective in decreasing the levels of both the pro- and anti-inflammatory cytokines, namely IL-8 and IL-10 respectively. Future studies should explore the role of intracanal medicaments on the levels of inflammatory mediators.

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