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(Article begins on next page)

## **Endothelial Dysfunction Marker Variation in Young Adults with Chronic Apical Periodontitis before and after Endodontic Treatment.**

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### **Abstract**

#### **Introduction**

Cardiovascular diseases are the leading cause of mortality worldwide. Apical periodontitis (AP) has been associated with an increased risk of cardiovascular diseases. A correlation has been shown between chronic AP and endothelial dysfunction (ED), but there is no evidence to indicate ED improves after endodontic treatment in patients with periapical lesions. The aim of this study was to investigate vascular and molecular markers of early ED before and after root canal treatment in young adults with chronic AP.

#### **Methods**

Twenty control subjects and 21 patients with AP were assessed at baseline. The AP patients were also evaluated 2 and 12 months post-treatment. Endothelial flow reserve was assessed via an endothelial function test, and enzyme-linked immunosorbent assays were used to evaluate plasma levels of proinflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor alpha; vasoconstrictor ED marker endothelin (ET)-1; circulating endothelial adhesion markers intercellular adhesion molecule 1 (ICAM-1)/CD54 and soluble vascular cellular adhesion molecule (sVCAM)-1/CD106; soluble CD14; and the endothelial leukocyte adhesion molecule (E-selectin).

#### **Results**

AP was associated with increased serum levels of ET-1, ICAM-1, E-selectin, IL-1, and sCD14, suggesting early vascular ED, with no macroscopic evidence of a reduction in endothelial flow reserve. Root canal treatment ameliorated inflammation and early ED, lowering plasma levels of IL-1, sCD14, ET-1, ICAM-1/CD54, and E-selectin to those of control subjects.

#### **Conclusions**

Our findings suggest that AP may drive early vascular ED and that the endodontic therapy of AP ameliorates early ED.

Cardiovascular diseases (CVDs) remain the leading cause of morbidity and mortality worldwide (1). The reduction of CVDs, through improved control of known risk factors and advances in cardiovascular medicine, has contributed significantly to the growing numbers of an aging population (2). However, CVDs are increasingly prevalent in the developing world because of changes in risk factor profiles (3).

Endothelial dysfunction (ED) is an imbalance between endothelium-derived vasodilators and vasoconstrictors (4). The vascular endothelium has multiple functions, including homeostasis of the microenvironment, nutrient exchange, hormone trafficking, fluid filtration, protection against thrombosis, host defense reactions (5), and modulation of the vascular tone by synthesizing and releasing an array of endothelium-derived relaxing and contracting factors. Endothelin (ET)-1 is the most potent endogenous vasoconstrictor (6). Relaxing factors include vasodilator prostaglandins, nitric oxide (NO), and endothelium-dependent hyperpolarization factors (7). Endothelial cell activation is defined by the endothelial expression of cell surface adhesion molecules, most notably intercellular adhesion molecule 1 (ICAM-1), soluble vascular cellular adhesion molecule 1 (sVCAM-1), and endothelial leukocyte adhesion molecule (also known as E-selectin), which facilitate the recruitment and attachment of

circulating leukocytes to the vessel wall (8). Both ED and endothelial cell activation are potentially useful predictors of CVDs.

In developed countries, the prevalence of apical periodontitis (AP) among 35- to 45-year-old adults is 34%–61% and increases with age (9). AP shows a similar immune response to that observed in marginal periodontitis (10) and may also contribute to systemic inflammation, increasing the levels of soluble CD14 (sCD14). This occurs as a consequence of lipopolysaccharide-induced activation of endothelial or epithelial cells that do not normally express membrane-bound CD14 in the sera of patients with marginal periodontitis (11). The inflammatory response may increase the risk of CVDs (12). Periradicular inflammatory apical lesions of endodontic origin have been associated with increased levels of serum C-reactive protein (CRP), as observed in periodontal disease (13, 14). Some studies have shown an increased risk of developing ischemic heart disease in subjects presenting with lesions of endodontic origin (15, 16) or pulpal inflammation (17, 18). An increase of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase (NOS), and interleukin (IL)-2, together with poor NO availability, has been observed in young male adults with AP and early signs of ED measured as endothelial flow reserve (EFR) by means of peripheral arterial tonometry (PAT) (19). Nevertheless, early preclinical ED is likely to be reversible (20), not only through lifestyle modifications and/or pharmacologic interventions, but also through the reversal of cardiovascular risk factors (21).

This study was designed to test the hypothesis that root canal treatment in patients with chronic AP could ameliorate the serum levels of ED and inflammatory markers. For this reason, the aim of the study was to investigate vascular and molecular markers of early ED before and after endodontic treatment.

## **Methods**

### **Study Population**

This study was performed in accordance with the Declaration of Helsinki and was authorized by the Città della Salute e della Scienza Ethical Committee (reference no. 0009323; CS2/510). Signed, written informed consent was obtained from all patients accepted for inclusion in this study. Consecutive dentate subjects with at least 1 periapical lesion (AP group) were enrolled according to the following inclusion criteria: below 35 years of age; normal weight; no medical history of diabetes or systemic, oncologic, or immune system diseases; no current immunosuppressive or corticosteroid drug treatment; no diagnosis of marginal periodontitis; and no other ongoing dental treatment during the 12-month study period. Control subjects (control group) were randomly enrolled from a general medical database from the same district and matched for age (matching range  $\pm 2$  years) with the AP group. Patients with any CVDs, as determined by a cardiologic checkup and electrocardiogram, were excluded from both groups before data collection. Structured patient medical and dental histories, demographics, and socioeconomic status were collected. Intraoral examination was performed using a 3.5 $\times$  Galilean loupes (Orascoptic, Middleton, WI). The pulpal and periradicular status of each patient was assessed with thermal and electric sensibility tests (Diagnostic Unit; Sybron Endo, Orange, CA), palpation, and percussion. Complete periodontal charting was recorded. Teeth with clinical signs of suspected AP were examined with phosphor sensor imaging plates for intraoral radiographs as recommended (22, 23) to assess the periapical status. Lesions of endodontic origin were defined by the loss of lamina dura and periodontal ligament enlargement of more than 2 mm at the largest diameter. Standardized periapical radiographs were obtained using the Rinn XCP alignment system (Rinn Corp, Elgin, IL) with customized silicone bite at diagnostic and follow-up visits. Clinical and radiologic data were analyzed, and diagnosis was formulated by 3 clinical assistant professors at the endodontics department. Examiners' performances were calibrated on the evaluation criteria by means of a case series presentation until interexaminer reliability could be expected; concordance between examiners was analyzed through the Fleiss  $\kappa$  score ( $\kappa > 0.70$ ).

### **Endothelial Function**

EFR was measured at the distal extremity of the upper limbs by PAT, a non-operator-dependent method that provides a reproducible index of endothelial-dependent vasodilation. ENDO-PAT2000

(Itamar Medical, Caesarea, Israel) was used with biosensors on the fingers to measure changes in vasal tone influenced by the endothelium. Modifications of vasal tone were produced by occlusion of the brachial artery for 5 minutes with a consequent hyperemic response; the contralateral arm was used as a control. EFR was measured at baseline in both groups and at 2 and 12 months postoperatively in the AP group.

### **Blood Sample Collection**

Blood samples were drawn into EDTA-K<sub>2</sub>-containing tubes (BDVacutainer; Bectin Dickinson Biosciences, Franklin Lakes, NJ) at the time of enrollment for all subjects and at 2 and 12 months after non-surgical root canal treatment for the AP patients. Plasma was isolated by centrifugation for 15 minutes at 2400g and immediately frozen at -80°C. Samples were stored at -80°C until they were analyzed. Hemolyzed samples were rejected, and a further withdrawal was made.

### **Biochemical Analysis: Enzyme-linked Immunosorbent Assay**

Enzyme-linked immunosorbent assays (R&D systems, Minneapolis, MN, and Sigma-Aldrich, St Louis, MO) were used to detect plasma concentrations of the proinflammatory cytokines IL-1, IL-6, and tumor necrosis factor alpha (TNF- $\alpha$ ); the powerful vasoconstrictor ED marker ET-1; the circulating endothelial adhesion marker, ICAM-1/CD54 and sVCAM-1/CD106; sCD14; and the endothelial leukocyte adhesion molecule E-selectin following the manufacturers' instructions. Enzyme-linked immunosorbent assays were performed by the same operator who was blinded to the sample origin. Intra-assay and interassay coefficients of variation were all <6%. All samples were measured in duplicate. Standard curves were constructed for each marker by plotting mean absorbance for the standard on the y-axis against concentration on the x-axis. A curve of best fit was plotted to determine the concentration of the analytes that were expressed as ng or  $\mu$ g/mL plasma.

### **Endodontic Treatment**

After local anesthesia and rubber dam isolation, access cavity and endodontic pretreatment were performed to create an adequate reservoir for irrigant solutions. Root canal scouting was performed. A mechanical glide path was created with ProGlider (Dentsply Maillefer, Baillagues, Switzerland) using an endodontic motor (X-Smart, Dentsply Maillefer) at the suggested settings at the working length (WL). The electronic WL was recorded with an apex locator (Diagnostic Unit) and checked with periapical radiographs.

Root canal shaping was performed with ProTaper Next X1-X2 (Dentsply Maillefer) at the WL. Apical patency was established and confirmed with a size 10 K-file 0.5 mm beyond the apex. Irrigation was performed with 5% sodium hypochlorite and 10% EDTA. Root canal filling was completed at the same session with sealer (Pulp Canal Sealer EWT; Kerr Endodontics, Orange, CA) and the Thermafil technique (Dentsply Maillefer) according to the manufacturers' instructions. The access cavity was sealed with a temporary filling (IRM; Dentsply International Inc, Milford, DE), and patients were scheduled for subsequent postendodontic restoration. Patients received a prescription for optional analgesics; no antibiotics or corticosteroids were recommended. The AP patients were followed up at 2 and 12 months after root canal treatment for biochemical analysis and at 12 months for clinical-radiologic reevaluation to assess the outcome of endodontic treatment (24). Patients' outcomes were categorized as healed, healing, and disease according to Friedman and Mor (25).

### **Statistical Analysis**

Continuous variables were presented as the mean  $\pm$  standard deviation. Normality was tested with the Shapiro-Wilk test. Parameter means were compared between AP patients and controls using unpaired Student *t* tests and among AP patients at different time points using 1-way repeated measure analyses of variance using the Greenhouse-Geisser correction to account for violation of the sphericity assumption. The linear correlation between variables was assessed with the Pearson correlation coefficient. AP patient parameter values at baseline and standard deviations of the difference after treatment were used to compute the minimum detectable difference between parameter levels after treatment, assuming a 5% significance level and 80% power. Results are

shown in Table 1. Statistical analyses were conducted using Stata Statistical Software, Release 15 (StataCorp LLC, College Station, TX).

### **Results**

The characteristics of 20 control subjects and 23 AP patients at baseline and 2 and 12 months posttreatment (2 AP patients were lost at follow-up at 12 months) are summarized in Table 2. Each patient in the AP group had 1 tooth affected by AP and received 1 root canal treatment; 19 patients (90.5%) showed a favorable outcome at 1 year (13 healed and 6 healing), whereas 2 were classified as diseased. The groups showed no statistically significant differences in the clinical parameters or markers of systemic inflammation, except for significantly higher concentrations of IL-1 and sCD14 in the AP group compared with the control subjects. Posttreatment serum concentrations of IL-1 and sCD14 were significantly lower than pretreatment concentrations in the AP patients and were comparable with those of the control subjects (Table 2). The mean values of endothelial reserve, measured as EFR, were similar in the control group, the AP group, and the AP group 2 and 12 months after treatment (Table 2).

Baseline concentrations of ET-1 were significantly higher in the AP patients compared with the control patients (Table 2), and these had decreased significantly at the 2- and 12-month posttreatment time points. ICAM-1/CD54 and E-selectin baseline concentrations were also significantly higher in the AP patients compared with control patients. Similarly, these were significantly reduced in the AP patients at the 2- and 12-month posttreatment time points (Table 2). sVCAM-1 levels were comparable between the groups at baseline and 2 months after treatment (Table 2). In the AP group, ED markers showed a positive trend of return to values comparable with those in controls after root canal treatment, except for the 2 nonresponder subjects (diseased) in whom the levels of both ED and inflammatory markers (Table 3) did not significantly differ from baseline during the observation period. This suggests that patients with persistent AP also had elevated ED and inflammatory markers.

Our results showed no correlation between the plasma concentrations of ET-1 and ICAM, E-selectin, or sCD14 (Fig. 1) or between sCD14 and ICAM-1 or E-selectin in control subjects (Fig. 2). A positive correlation was observed between the plasma concentrations of ET-1 and ICAM, E-selectin, and sCD14 (Fig. 1) as well as between sCD14 and ICAM-1 and E-selectin in the AP patients at baseline (Fig. 2). This positive correlation had disappeared by the 2- or 12-month posttreatment follow-ups (Figs. 1 and 2).

### **Discussion**

A prerequisite for cardiovascular atherosclerotic disease is ED, which forms a potential basis for the disruption of vascular function. Endothelial function serves as an excellent surrogate marker of cardiovascular events in humans and has attracted much attention in the clinical setting (26). Several methods have been used to assess endothelial function using flow-mediated dilation of the brachial artery or vasodilatory responses to intrabrachial artery infusion of acetylcholine. Furthermore, reduced NO levels are thought to be a reliable indicator of ED (27). An increase of asymmetric dimethylarginine (an endogenous inhibitor of NOS) and IL-2 concentrations, combined with poor NO availability, have been described in young male adults with AP and early signs of ED, measured as EFR at the level of the distal extremity in the upper limbs by means of PAT (19). However, in this study, we did not observe any difference in EFR levels between the AP and control groups. We may extrapolate that biomarker modification occurred before a detectable basal tone alteration in this study population.

A large body of experimental and clinical data has implicated the endothelin system in early-stage ED (8). In particular, increased expression of ET-1, the most potent endogenous vasoconstrictor and proinflammatory peptide (6), exacerbates diabetes-induced ED (28), causing a decrease in endothelial NOS expression, an increase in vascular oxidative stress, and a decrease in antioxidant capacity. In this study, ET-1 expression was higher in the AP group than the control group at baseline. This suggests that apical lesions may lead to an increase of serum ET-1 that, in turn, could drive ED. Notably, the successful treatment of apical lesions was

associated with a reduction in serum ET-1 levels at 2- and 12-month assessments after root canal treatment.

Furthermore, there is an established link between ED and endothelial cell activation (29). An alternative method to assess endothelial function involves the measurement of circulating biomarkers that are associated with endothelial activation and that may be predictive of CVD. It has been shown that E-selectin (30), sVCAM-1, and ICAM-1(27) can be used as markers to predict mortality and subclinical atherosclerosis. As a predictor of cardiovascular events, atherosclerosis often develops subclinically over prolonged periods of time.

If left unchecked, ED and endothelial cell activation can lead to atherosclerosis and vascular disease by increasing vasoconstriction, smooth muscle cell proliferation, platelet aggregation, leukocyte adhesion, low-density lipoprotein oxidation, and matrix metalloproteinase activation (29). We found that only ICAM-1 and E-selectin, as subclinical atherosclerosis markers, were markedly higher in AP patients compared with controls. After root canal treatment, concentrations of these adhesion molecules in responding subjects returned to levels equivalent to those observed in controls.

The expression of these molecules on tissue endothelium is controlled via the cytokine-mediated endothelial activation pathway. This pathway is initiated as a result of a cell-mediated immune response to infectious agents (31) and/or through the action of ET-1 (32). Indeed, cardiovascular risk factors, such as hypercholesterolemia, smoking, and oxidative stress, are important mediators of ED. Proinflammatory cytokines, such as TNF- $\alpha$  and IL-6, as well as turbulent flow and advanced glycation end products are important mediators of endothelial cell activation (8).

In this study, the AP patients had higher serum concentrations of IL-1 and sCD14 than control patients, whereas the serum concentrations of IL-6 and TNF- $\alpha$ , nontraditional cardiovascular risk factors, did not differ between the AP and control groups. CRP was avoided in this study because levels can be elevated by noncardiovascular factors (33), and the addition of CRP to risk prediction models for intermediate-risk individuals improves risk stratification (34).

One of the limitations of this study is the relatively small sample size. Consequently, when interpreting these findings, consideration should be given to the estimates of correlations and associations because these may differ when assessed in other groups of patients.

Moreover, the control group should have been followed up at 2 and 12 months. However, because there were no clinical/radiographic evidence of onset of periapical lesions and no change in lifestyle and medical history of these subjects, this group was considered unchanged during the year and being representative of a healthy population. For this reason, it was considered ethically correct to avoid further blood sample collections for biochemical analysis.

In conclusion, our data suggest that AP may drive early vascular ED, showing as increased serum levels of ET-1, ICAM-1, and E-selectin adhesion molecules and inflammatory IL-1 and sCD14, without any macroscopic evidence of a reduction in EFR. Moreover, the treatment of AP ameliorates early ED because the significant increase of these molecules has been abolished at 2 and 12 months posttreatment.

### **Acknowledgments**

The authors deny any conflicts of interest related to this study.

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