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Increased Root Canal Endotoxin Levels are Associated with Chronic Apical Periodontitis, Increased Oxidative and Nitrosative Stress, Major Depression, Severity of Depression, and a Lowered Quality of Life

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Abstract Evidence indicates that major depression is accompanied by increased translocation of gut commensal Gram-negative bacteria (leaky gut) and consequent activation of oxidative and nitrosative (O&NS) pathways. This present study examined the associations among chronic apical periodontitis (CAP), root canal endotoxin levels (lipopolysaccharides, LPS), O&NS pathways, depressive symptoms, and quality of life. Measurements included advanced oxidation protein products (AOPP), nitric oxide metabolites (NOx), lipid peroxides (LOOH), −sulfhydryl (SH) groups, total radical trapping antioxidant parameter (TRAP), and paraoxonase (PON)1 activity in participants with CAP, with and without depression, as well as healthy controls (no depression, no CAP). Root canal LPS levels were positively associated with CAP, clinical depression, severity of depression (as measured with the Hamilton Depression Rating Scale (HDRS) and the Beck Depression Inventory) and O&NS biomarkers, especially NOx and TRAP. CAP-related depression was accompanied by increased levels of NOx, LOOH, AOPP, and TRAP. In CAP participants, there was a strong correlation ($r = 0.734$, $p < 0.001$) between root canal LPS and the HDRS score. There were significant and positive associations between CAP or root canal endotoxin with the vegetative and physiosomatic symptoms of the HDRS as well as a significant inverse association between root canal endotoxin and quality of life with strong effects on psychological, environmental, and social domains. It is concluded that increased root canal LPS accompanying CAP may cause depression and a lowered quality of life, which may be partly explained by activated O&NS pathways, especially NOx thereby enhancing

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hypernitrosylation and thus neuroprogressive processes. Dental health and "leaky teeth" may be intimately linked to the etiology and course of depression, while significantly impacting quality of life.

Keywords Depression · LPS · Endotoxin · Inflammation · Oxidative and nitrosative stress · Antioxidants · Chronic apical periodontitis

Introduction

Research on the pathophysiology of clinical depression has highlighted activated immune-inflammatory, oxidative and nitrosative stress (O&NS) pathways [1, 2]. Immune activation and chronic mild inflammation in depression are indicated by increased plasma levels of positive acute phase proteins, e.g., haptoglobin and C-reactive protein (CRP), and increased levels of T helper cells (Th1) and M1 macrophagic cytokines [1, 3, 4]. Lowered levels of endogenous antioxidants and antioxidant enzymes, including high-density lipoprotein (HDL) cholesterol, vitamin E, paraoxonase (PON)1, coenzyme Q10, and zinc, are often observed in clinical depression and animal models of depression [2, 5–10]. Immune activation and chronic inflammatory processes are accompanied by increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as superoxide, peroxides, nitric oxide (NO), and peroxynitrites [10–12]. When the pro-oxidant/antioxidant ratio increases, damage by O&NS processes may cause lipid peroxidation, oxidative damage to membrane lipids and anchorage molecules, protein oxidation and hypernitrosylation, i.e., binding of NO (nitroso-) to proteins [11, 12]. Increased levels of peroxides, malondialdehyde (indicating lipid peroxidation), advanced oxidation protein products (AOPP), indicating protein oxidation, NO production, and NO-binding to proteins are all evident in clinical depression [2, 7, 10, 12–14].

Alterations in the gut-brain-axis following increased gut permeability (leaky gut) may contribute to activated immune-inflammatory and O&NS pathways and subsequently to depression symptoms [15, 16]. One mechanism explaining this pathway is translocation of Gram-negative bacteria causing increased endotoxin or lipopolysaccharide (LPS) levels in blood, which subsequently may activate Toll-like receptors (TLR) 2/4 leading to increased production of Th1 and M1 macrophagic cytokines as well as ROS/RNS [17, 18]. Clinical depression is accompanied by increased serum levels of immunoglobulin (Ig)A and IgM directed against LPS and antigens of different Gram-negative gut, commensal bacteria, including *Hafnia alvei*, *Pseudomonas aeruginosa*, *Morganella morganii*, *Proteus mirabilis*, *Pseudomonas putida*, *Citrobacter koseri*, and *Klebsiella pneumoniae* [19, 20]. Indicants of bacterial translocation in clinical depression are also associated with signs of inflammation and O&NS, including lipid peroxidation and hypernitrosylation [16].

Bacterial translocation of Gram-negative bacteria may also be induced through periodontal disease, including chronic apical periodontitis (CAP) [21, 22], which is a chronic inflammatory disorder of the periradicular tissues caused by bacterial invasion of the apex of the tooth root [23]. CAP not only causes local tissue inflammatory destruction, but also systemic inflammatory responses, which may ultimately predispose toward systemic disease, including cardiovascular disorder [22, 24–26]. There is a significant association between periodontitis and depression [27]. In a nationwide population-based study periodontitis was a significant risk factor for depression with an adjusted hazard ratio of 1.73 [28]. Moreover, lowered self-esteem and higher levels of stress have been reported in subjects with periodontal disease [29–31]. Gram-negative and -positive bacteria, including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Pseudomonas aeruginosa*, *Bacteroides forsythus*, *Campylobacter rectus*, *Peptostreptococcus micros*, *Staphylococcus intermedius*, and *Treponema* sp. play an important role in the pathophysiology of periodontitis and CAP [22, 32, 33]. There are, however, no data whether LPS levels in the root canals of patients with CAP are associated with depression and systemic O&NS processes.

The aims of this study were to delineate whether root canal endotoxin in patients with CAP is increased in patients with depression as compared to those without depression and controls; whether there are significant associations between root canal endotoxin contents and severity of illness, quality of life and O&NS, including hydroperoxide, AOPP, NO production, and antioxidant defenses. The a priori hypotheses are that increased root canal endotoxin levels are associated with depression, severity of depression, a lowered quality of life, increased O&NS, and lowered antioxidant defenses.

Participants and Methods

Participants

We recruited subjects with CAP with and without depression, diagnosed according to DSM-IV-TR, who attended the Dental Clinic of the School of Dentistry at the Fluminense Federal University, Nova Friburgo, RJ, Brazil. Healthy volunteers were sampled from the same catchment area. To be included, participants had to be over 18 years of age or older and be able to read and write Portuguese language. CAP participants were only included if they planned to have their teeth extracted at this dental clinic. CAP patients with depression were excluded when a current or lifetime axis-I diagnoses, other than major depression, was present including autism, schizophrenia, cognitive disorders, and substance abuse. CAP patients without

depression and healthy controls (no CAP, no depression) were excluded if a lifetime or current diagnosis of axis-I diagnosis was evident, including major depression, dysthymia, bipolar disorder, autism, schizophrenia, cognitive disorders, and substance abuse. Both CAP and healthy subjects were excluded if any major medical disorder was present, including immune and autoimmune disorders, diabetes, lupus erythematosus, colitis ulcerosa, and Chrohn's disease.

Based on the inclusion criteria, we recruited 59 subjects. Due to the abovementioned exclusion criteria, we excluded some subjects with CAP and depression, namely one with autism, one with schizophrenia, three with cognitive disorders, and two with substance abuse. We also excluded controls without depression and CAP, namely two with a current diagnosis of diabetes, two with immune disorders, and one with substance abuse. As a consequence, the final study sample comprised 23 normal controls and 24 subjects with depression. The study was approved by the Ethics Committee of the Federal Fluminense University, Nova Friburgo, RJ, Brazil (No. 1.555.226). Written informed consent was obtained from all participants.

Measurements

All participants were assessed by a senior psychiatrist and senior dentist using semi-structured interviews. Intraoral clinical and radiographic exams were conducted in order to identify the presence of extensive tooth cavities in demand of extraction and presence of CAP, respectively. All participants completed a questionnaire consisting of sociodemographic data, smoking history, and data on family history of psychiatric disease. The psychiatric interview consisted of (a) structured clinical interview, clinical version (SCID-I) to make the diagnosis of current/lifetime axis-I disorders according to DSM-IV-TR criteria. (b) 17-item Hamilton Depression Rating Scale (HDRS) in a validated Portuguese translation [34]. We also examined three different subscores, namely sum of items 1–3, 7, and 17 reflecting "core depressive" symptoms (HDRSd); sum of items 4–6, 8, and 16 reflecting "vegetative symptoms" (HDRSv), and sum of items 11–15, reflecting "physio-somatic symptoms" (HDRSps). (c) Beck Depression Inventory (BDI) in a validated Portuguese translation adapted to the Brazilian population [35]. (d) Quality of life was measured using the WHO Quality of Life (WHOQoL)-BREF in a validated Portuguese translation [36]. We computed the raw scores on the four WHOQol-BREF domains (namely (1) physical health, (2) psychological health, (3) social relationships, and (4) environment) rather than the transformed scores (which convert the lowest and highest scores to 0 and 100, respectively). Total health-related Qol was estimated by summing of the raw scores of the four domains. (e) We employed the Alcohol, Smoking, and Substance Involvement Screening Test (ASSIST) to screen for the use of hypnotics and alcohol, translated and adapted to Portuguese by Henrique et al. [37]. In order to assess severity of tobacco dependence, we used the Fagerstrom Test for Nicotine Dependence in a Portuguese translation [38]. Number of pack years was calculated as the number of cigarettes smoked per day multiplied by number of years smoked and divided by 20 (one pack has 20 cigarettes). As a surrogate but adequate measurement of body mass index we measured waist circumference.

Plasma O&NS Biomarkers

After an overnight fast we collected blood at 8:00 a.m., the same day as teeth extractions, for the assay of O&NS and antioxidant biomarkers, including AOPP, hydroperoxides (LOOH), nitric oxide (NO) metabolites (NOx), sulfhydryl (−SH) groups, PON1 total activity, and total radical trapping antioxidant parameter (TRAP). To measure protein oxidation, AOPP in plasma was quantified—using the method described by Hanasand et al. [39] in a microplate reader, PerkinElmer®, model EnSpire (Waltham, MA, EUA) at a wavelength of 340 nm. AOPP concentration was expressed in micromoles of equivalent chloramine T. LOOH were determined according to an adaptation of the technique described by Gonzales-Flecha et al. [40] and Panis et al. [41]. This method uses the compound tert-butyl hydroperoxide to start a lipid chain reaction that can be detected by photon emission during the formation of lipid hydroperoxides. Readings were performed in a Glomax luminometer (TD 20/20 Turner Designers, E.U.A.) over 1 h at 1 reading/s. Results were expressed as relative units of light. NO levels were assessed indirectly by determining the plasma nitrite concentration using an adaptation of the technique described Navarro-Gonzálvez et al. [42]. This method is based on the reduction of the nitrate present in the sample to nitrite by oxidation-reduction reactions mediated by the system cadmium-copper reagent. Thereafter, Griess reagent was added to induce diazotization, forming a colored complex and subsequent detection at 540 nm. The quantification of NOx was made in a microplate reader Asys Expert Plus, Biochrom® (Holliston, MA, USA). The nitric oxide metabolites concentrations were expressed in micromoles. Sulfhydryl groups from proteins were evaluated by the method described by Hu [43], which is based on the reaction of 5,5-dithiobis-2 nitrobenzoic acid (DTNB) with sulfhydryl groups. Determination was conducted in a spectrophotometer Helios α, Thermo Spectronic® (Waltham, MA, USA) at 412 nm. Results are expressed as micromoles per milligram of plasmatic protein. Total plasmatic activity of PON1 was determined by the method described by Richter et al. [44]. The rate of hydrolysis of phenyl acetate was determined in a microplate reader EnSpire, PerkinElmer® (Waltham, MA, USA) at 270 nm and the temperature maintained at 25 °C. Measures were recorded every 4 min, each of 15 s. The activity was

expressed in units per milliliter based on the phenyl acetate molar extinction coefficient of 1.31 mMol/L cm^{-1}. TRAP was evaluated according to the method described by Repetto et al. [45] in microplate reader Victor X-3, PerkinElmer® (Waltham, MA, USA). Experimental conditions were running time of 25 min, response range from 300 to 620 nm and a temperature of 30 °C. This method detects hydro- and/or lipo-soluble antioxidants in serum. The results were expressed in micromoles trolox. All analytes were assayed in one and the same run by the same technician, who was blinded to the source of the clinical data. The intra-assay CV values were all <6%.

Procedures

Identified teeth were extracted according to a standard surgical protocol. Briefly, following mouth wash with antiseptic solution (Listerine - Johnson & Johnson do Brasil Indústria e Comércio de Produtos para Saúde Ltda, SP, Brazil) extra oral asepsis was carried out with iodine alcohol (Povidine - Johnson & Johnson do Brasil Indústria e Comércio de Produtos para Saúde Ltda, SP, Brazil). Anesthesia was performed using articaine hydrochloride and epinephrine 1:100.000 (DFL Indústria e Comércio S.A, RJ, Brazil). Subsequently, syndesmotomy was performed using Sindestome (Duflex, S.S.White, RJ, Brazil) and the tooth was extracted using forceps (Duflex, S.S.White, RJ, Brazil). Curettage of the peri-apical lesion and alveolus was performed with surgical curettes. Hemostasis was performed with gauze followed by suture with needle holder (Duflex, S.S.White, RJ, Brazil) and suture needle line 4–0 needle CT 1/2 1.7 cm (Duflex, S.S.White, RJ, Brazil). The extraction of the pulp was performed according to standardized methods as previously described by us [46, 47]. In short: the sampling of the infection pool, which includes bacteria, endotoxins and remnants of pulp tissue, is performed by placement of a sterile paper point inside the root canal for 1 min. Consequently, the paper point is placed in a 1.5-mL plastic tube (sterile and free of endotoxins) and the sampling specimen is reconstituted with 1 mL of limulus amebocyte lysate (LAL) water (LAL Reagent Water), whereby the extraction of the infection pool from the paper point is mechanically performed by agitation and sonication from the samples in a vortex for 60 s. The root canal sample specimens are then frozen at −80 °C until assayed for endotoxin.

Endotoxin Assay

A turbidimetric test (Kit KQCL, BioWhitaker, Inc., Walkersville, MD) and the limulus amebocyte lysate (LAL) technique were used to measure endotoxin levels. The test procedure was performed following the manufacturer's instructions. For the calculation of the amount of endotoxins in root canal samples, a standard curve was plotted by using endotoxins supplied in the kit. The analyses were carried out in triplicate for each dilution in the concentration range as provided by the manufacturer. These results were used to produce a linear regression curve for the determination of linear and angular parameters. Additionally, the correlation coefficient and replicates relative standard deviation were determined. A 96-well microplate (Corning Costar, Cambridge, MA) was placed on a heating block at 37 °C and maintained at this temperature throughout the assay. Next, the endotoxin samples were suspended in 1-mL LAL water, supplied with the kit, and agitated in a vortex for 60 s, thereafter being serially diluted to a concentration of 10^{-1}. Immediately afterwards, 100 μL of the blank, followed by the standard endotoxin solutions at different concentrations and 100 μL of the samples were added in duplicate in the 96-well microplate. Endotoxin absorbance was measured using an enzyme-linked immunosorbent assay plate reader (Ultramark; BioRad Laboratories, Hercules, CA) at 340 nm. The mean absorbance value of the standard solutions was directly proportional to the concentration of endotoxins and the concentration was determined from the standard curve. The intra-assay CV values were <10%. Previously, we compared different tests using the LAL principle for the analysis of endotoxins in root canal contents and we concluded that quantitative kinetic-turbidimetric (used here) and kinetic-chromogenic LAL methods are best fitted for the analysis of endotoxins in root canal infection, both being more precise and allowing better reproducibility compared with the endpoint-QCL assay [48].

Statistical Analysis

We used analyses of variance (ANOVAs) to check differences in scale variables among study groups and analyses of contingency tables, the X^2 test, to assess associations between nominal variables. Pearson's product moment, Spearman's rank order, and point-biserial correlation analyses were employed to assess correlations between two sets of variables. We employed multivariate general linear model (GLM) analyses to delineate the multivariate effects of selected explanatory variables on dependent variables. Thus, we used root canal LPS and/or the O&NS biomarkers as dependent variables and the clinical diagnosis of depression as explanatory variables, while adjusting for relevant background variables including age, sex, and smoking. We also used severity of depression or the WHOQoL-BREF data as dependent variables and the clinical diagnosis of periodontitis or root canal LPS levels as explanatory levels while adjusting for the relevant background variables. Consequently, tests for between-subjects effects were employed to delineate the univariate effects of significant predictor variables on each of the dependent variables. Stepwise automatic regression analysis or univariate GLM analyses were used to assesses the effects of

selected explanatory variables on one dependent variable. We used Ln or square root transformations to normalize the data distribution (tested with the Kolmogorov-Smirnov test) of biomarkers where needed, namely LPS, AOPP, NOx, and LOOH. To provide an integrative O&NS index, a z unit weighted composite score was computed, and consequently raw values of AOPP, LOOH, and NOx were converted into z scores and added up as zO&NS = zAOPP + zLOOH + zNOx. All results of regression analyses were checked for collinearity using collinearity statistics, VIF, and tolerance. All statistical analyses were performed using IBM SPSS windows version 22. Tests were two-tailed and a p value of 0.05 was used for statistical significance.

Results

Table 1 shows the sociodemographic, clinical, and biomarker data of all participants divided into those with increased root canal LPS (as determined with the median-split method) versus those with lower LPS values. No p corrections were employed to interpret the results presented in Table 1 as these univariate tests together with the correlation matrices between the variables were used to delineate the explanatory and background variables, which were employed in the ultimate multivariate GLM analyses as independent determinants of the dependent variables. Nevertheless, Table 1 shows that there were no significant differences in age, sex distribution, waist circumference, hypertension, and smoking between the study groups. Only two subjects smoked between 11 and 20 cigarettes/day, while the others (n = 9) smoked 10 or less cigarettes/day. Participants with higher root canal LPS levels showed increased prevalence of CAP and depression and increased HDRS (and subscores) and BDI values and lowered WHOQoL-BREF (and domain) values. In the same participants, there was a trend toward increased AOPP values and increased LOOH, NOx, zO&NS, and TRAP values. There were no differences in −SH groups and PON1 values. In the total study group, there were significant correlations between root canal LPS and HDRS (r = 0.799, p < 0.001, n = 47); BDI (r = 0.759, p < 0.001, n = 47); and WHOQoL-BREF score (r = −0.529, p < 0.001, n = 46). In subjects with CAP, there were significant correlations between root canal LPS and HDRS (r = 0.734, p < 0.001, n = 34) and the BDI (r = 0.704, p < 0.001, n = 34).

Table 2 displays the results of a multivariate GLM analyses with LPS and the six O&NS biomarkers as dependent variables and clinical depression as primary explanatory variable while adjusting for age, sex, and smoking. All four explanatory variables had significant effects on the biomarkers. Tests for between-subject effects and the estimated marginal mean values show that clinical depression was significantly associated with increased root canal LPS, plasma AOPP, NOx, LOOH, and TRAP values, while there were no significant effects of –SH groups and PON1 activity. There were sex-related differences in AOPP, PON1, and TRAP, while age had a significant effect on LPS and LOOH (positive) and –SH groups (negative). Smoking had a small, although significant, effect on LOOH and NOx (positive). Univariate GLM analysis showed significant effects (F = 7.49, df = 5/39, p < 0.001) of clinical depression (F = 12.97, df = 1/39, p = 0.001); age (F = 6.05, df = 1/39, p = 0.018); and smoking (F = 9.72, df = 1/39, p = 0.003), but not CAP (F = 2.18, df = 1/39, p = 0.148) and sex (F = 3.18, df = 1/39, p = 0.082) on zO&NS. This O&NS index was significantly higher in participants with depression (mean ± SD = 1.98 ± 0.58 in z values) than in subjects without depression (−0.45 ± 0.39 in z values).

We have examined other putative explanatory variables in this multivariate GLM analysis but none were significant, including waist circumference (F = 0.72, df = 7/30, p = 0.659); hypertension (F = 1.30, df = 7/32, p = 0.280); ASSIST alcohol (F = 1.68, df = 7/32, p = 0.149); ASSIST hypnotics (F = 1.64, df = 7/32, p = 0.161); Fagerstrom total score (F = 1.72, df = 7/33, p = 0.138); and cigarette month–packs/year (F = 0.70, df = 7/33, p = 0.600).

Table 3 shows the results of two different multivariate GLM analyses with the O&NS biomarkers as dependent variables and CAP or root canal LPS levels as explanatory variables, while adjusting for background variables. We found that CAP did not have significant effects on the O&NS biomarkers, while root canal LPS was a significant predictor variable. Tests for between-subject effects showed that root canal LPS was associated with increased NOx, TRAP, and AOPP, after adjusting for sex, age, and smoking. Univariate GLM analysis showed that root canal LPS was also associated with an increased zO&NS index (F = 28.90, df = 1/40, p = 0.004). We have also examined the combined effects of depression and CAP on the six O&NS biomarkers. Multivariate GLM analysis showed that depression (F = 6.52, df = 6/32, p < 0.001) (and age, sex, and smoking), but not CAP (F = 1.99, df = 6/32, p = 0.096), had a significant effect on the six O&NS biomarkers. We have also examined the effects of CAP and depression on LPS. Univariate GLM analysis showed that 92.6% of the variance in LPS levels (F = 287.46, df = 2/44, p < 0.001) was explained by CAP (F = 76.49, df = 1/44, p < 0.001) and depression (F = 138.85, df = 1/44, p < 0.001). Root canal LPS was significantly higher in depressed subjects (206.3 ± 14.4 EU/mL) than in those without depression (25.0 ± 10.3 EU/mL) and higher in those with CAP (141.2 ± 14.4 EU/mL) than in those without (90.5 ± 16.4 EU/mL).

Table 4 shows the results of three multivariate GLM analyses with the WHOQol-BREF, HDRS, and BDI as dependent variables and CAP or LPS as explanatory variables. Age, sex, smoking, and waist circumference were not significant in this

Table 1 Sociodemographic, clinical, and biomarker data in subjects with increased root canal endotoxin levels versus those with lower root canal endotoxin levels

Variables	LPS < 119.4 EU/mL	LPS ≥ 119.4 EU/mL	F/X^2	df	*p* value
Age (years)	41.4 (20.6)	45.9 (13.1)	0.80	1/45	0.375
Sex (♀/♂)	13/10	15/9	0.17	1	0.676
Chronic apical peridontitis (no/yes)	13/10	0/24	FEPT	–	<0.001
Major depression (no/yes)	22/1	1/23	FEPT	–	<0.001
Waist circumference (cm)	89.5 (16.6)	91.3 (11.9)	0.18	1/43	0.677
Education (years)	9.9 (4.1)	6.6 (3.9)	6.66	1/38	0.014
Hypertension (no/yes)	18/5	13/11	3.04	1	0.081
Smoking (no/yes)	19/4	15/9	2.37	1	0.123
HDRS	3.1 (1.8)	14.0 (5.6)	78.78	1/45	<0.001
BDI	2.9 (6.3)	17.5 (10.6)	32.60	1/45	<0.001
HDRSd	2.3 (0.8)	4.5 (2.5)	15.37	1/45	<0.001
HDRSv	0.3 (1.1)	4.6 (2.5)	58.05	1/45	<0.001
HDRSps	0.04 (0.2)	2.4 (1.9)	33.18	1/45	<0.001
Total WHOQol-BREF	94.9 (10.8)	78.0 (14.7)	19.37	1/44	<0.001
WHO-Physical	29.3 (4.7)	23.7 (5.4)	14.80	1/45	<0.001
WHO-Psychological	25.6 (2.4)	20.4 (5.6)	17.17	1/45	<0.001
WHO-Social relationships	12.0 (1.8)	10.2 (2.3)	9.57	1/44	0.003
WHO-Environmental	28.0 (4.2)	23.8 (5.0)	9.53	1/45	0.003
Endotoxin (EU/mL)[a]	21.5 (31.1)	232.2 (63.8)	51.34	1/45	<0.001
AOPP (:M) [a]	89.4 (37.0)	169.5 (220.1)	3.05	1/44	0.088
LOOH (RLU 10E6) [a]	860 (87)	967 (166)	6.49	1/43	0.015
NOx (:M) [a]	7.8 (3.8)	10.5 (5.1)	5.31	1/44	0.026
−SH groups (:M)	378.4 (53.2)	385.6 (52.0)	0.22	1/44	0.643
TRAP (:M Trolox)	759.4 (131.6)	928.4 (158.6)	15.30	1/43	<0.001
PON1 (U/mL)	161.7 (44.6)	176.1 (39.3)	1.33	1/43	0.256
Z LOOH + AOPP + NOX	−0.98 (1.48)	0.93 (2.46)	9.79	0/43	<0.001

All results are shown as mean (SD). All results of analyses of variance (ANOVA), or analyses of contingency tables (X^2) or FEPT: Fisher's exact probability test. All results of analyses of variance (ANOVA), or analyses of contingency tables (X^2) or FEPT

[a] Processed in Ln or square root transformation

HDRS Hamilton Depression Rating Scale; *BDI* Beck Depression Inventory; *WHOQoL-BREF* WHO Quality of Life-BREF; *HDRSd* HDRS subscore "core depressive symptoms," that is sum of items 1, 2, 3, 7, and 17; *HDRSv* HDRS subscore "vegetative symptoms," that is sum of items 4, 5, 6, 8, and 16; *HDRSps* HDRS subscore "physio-somatic symptoms," that is sum of items 11, 12, 13, 14, and 15; *AOPP* advanced oxidation protein products; *LOOH* hydroperoxide; *NOx* nitric oxide metabolites; *−SH groups* −sulfhydryl groups; *TRAP* total radical trapping antioxidant parameter; *PON1* paraoxonase total activity

regression but hypertension was. Therefore, we have entered hypertension as an additional explanatory variable. There were significant effects of CAP together with hypertension on the clinical rating scale scores. Tests for between-subject effects and estimated marginal mean values showed that CAP was significantly and positively associated with HDRS and BDI and inversely with WHOQoL-BREF. Hypertension was significantly and positively associated with HDRS score (see also estimated marginal mean values). Regression #2, Table 4 shows that there was a significant effect of root canal LPS on the clinical rating scale scores (hypertension was not significant in this regression). Tests for between-subject effects showed that root canal LPS was significantly and positively associated with HDRS and BDI and negatively with WHOQoL-BREF. Regression #3, Table 4 shows that NOx (but not other O&NS biomarkers) had a significant effect on the three rating scales and that increased NOx was associated with raised BDI and HDRS values.

Table 5 shows the results of three multivariate GLM analyses with the three HDRS subscales as dependent variables and CAP or root canal LPS as explanatory variables. CAP and especially increased root canal LPS were strongly associated with the HDRS subscores, while tests for between-subject effects showed significant effects of root canal LPS on all three subscales, especially the vegetative and physio-somatic subscales. Multivariate GLM analysis #3 showed that NOx

Table 2 Results of multivariate GLM analyses with root canal endotoxin levels and oxidative and nitrosative stress and antioxidant biomarkers as dependent variables

Tests	Dependent variables	Explanatory variables	*F*	df	*p* value	Partial eta squared
Multivariate	All 7 biomarkers	Depression	19.40	7/32	<0.001	0.809
		Sex	4.78	7/32	0.001	0.511
		Age	10.62	7/32	<0.001	0.699
		Smoking	2.93	7/32	0.017	0.391
Between-subject effects	Endotoxin	Depression (+)	57.82	1/38	<0.001	0.603
		Age (+)	18.31	1/38	<0.001	0.325
	AOPP	Depression (+)	4.08	1/38	0.050	0.097
		Sex (M > F)	8.88	1/38	0.005	0.189
	SH groups	Age (−)	26.16	1/38	<0.001	0.408
	LOOH	Depression (+)	4.55	1/38	0.039	0.107
		Smoking (+)	4.43	1/38	0.042	0.104
		Age (+)	8.34	1/38	0.006	0.180
	PON1	Sex (F > M)	4.43	1/38	0.042	0.104
	NOX	Depression (+)	6.16	1/38	0.018	0.139
		Smoking (+)	5.05	1/38	0.031	0.117
	TRAP	Depression (+)	22.70	1/38	<0.001	0.374
		Sex (M > F)	5.07	1/38	0.030	0.118
Estimated marginal means (SE) in *z* values						
Biomarkers		Controls		Depression		
Endotoxin (*z* value)		−0.63 (0.14)		+0.74 (0.13)		
LOOH (*z* value)		−0.18 (0.20)		+0.38 (0.19)		
NOx (*z* value)		−0.20 (0.50)		+0.50 (0.21)		
TRAP (*z* value)		−0.52 (0.19)		+0.65 (0.18)		
AOPP (*z* value)		−0.12 (0.21)		+0.43 (0.20)		

AOPP advanced oxidation protein products, *LOOH* hydroperoxide; *NOx* nitric oxide metabolites; *−SH groups* −sulfhydryl groups; *TRAP* total radical trapping potential; *PON1* paraoxonase total activity; *M/F* male/female

and TRAP had significant effects on the three subscales, NOx on HDRSd and TRAP on the HDRSv and HDRSps subscales.

Table 6 shows the results of two multivariate GLM analyses with the four WHOQol-BREF domains as dependent variables, with CAP or root canal LPS as explanatory variables. CAP was significantly associated with lowered ratings on psychological, environment and social domains, while root canal LPS was significantly and inversely associated with the four domains.

Table 3 Results of multivariate GLM analyses with the oxidative and nitrosative stress and antioxidant data as dependent variables

Tests	Dependent variables	Explanatory variables	*F*	df	*p* value	Partial eta squared
Multivariate #1	AOPP, −SH, LOOH, PON1, NOx, TRAP	Periodontitis	1.70	6/33	0.151	0.236
		Smoking	1.95	6/33	0.102	0.262
		Sex	4.94	6/33	0.001	0.473
		Age	6.56	6/33	<0.001	0.544
Multivariate #2	AOPP, −SH, LOOH, PON1, NOx, TRAP	Endotoxin	4.05	6/33	0.004	0.424
		Smoking	1.70	6/33	0.152	0.236
		Sex	5.19	6/33	0.001	0.485
		Age	7.43	6/33	<0.001	0.575
	NOx	Endotoxin (+)	4.16	1/38	0.048	0.099
	TRAP	Endotoxin (+)	14.73	1/38	<0.001	0.279
	AOPP	Endotoxin (+)	4.50	1/38	0.041	0.106

AOPP advanced oxidation protein products, *LOOH* hydroperoxides, *NOx* nitric oxide metabolites, *−SH groups* −sulfhydryl groups, *TRAP* total radical trapping antioxidant parameter, *PON1* paraoxonase total activity

Table 4 Results of multivariate GLM analyses with the Hamilton Depression Rating Scale (HRDS). Beck Depression Inventory (BDI), and the WHO Quality of Life (Qol)-BREF scores as dependent variables and root canal endotoxin levels, chronic apical periodontitis (CAP) or biomarkers as explanatory variables

Tests	Dependent variables	Explanatory variables	*F*	df	*p* value	Partial eta squared
Multivariate #1	WHO, BDI, HDRS	CAP	5.93	3/41	0.002	0.302
		Hypertension	4.34	3/41	0.010	0.241
Between-subject effects	WHO	CAP (−)	10.14	1/42	0.003	0.190
	BDI	CAP (+)	10.08	1/43	0.003	0.190
	HDRS	CAP (+)	17.03	1/43	<0.001	0.284
		Hypertension (+)	5.03	1/43	0.030	0.105
Multivariate #2	WHO, BDI, HDRS	Endotoxin	25.45	3/42	<0.001	0.645
Between-subject effects	WHO	Endotoxin (−)	15.96	1/44	0.001	0.266
	BDI	Endotoxin (+)	36.53	1/44	<0.001	0.454
	HDRS	Endotoxin (+)	79.27	1/44	<0.001	0.642
Multivariate #3	WHO, BDI, HDRS	NOx	2.84	3/40	0.050	0.176
		Hypertension	4.10	3/40	0.013	0.235
Between-subject effects	BDI	NOx (+)	7.44	1/42	0.009	0.151
	HDRS	NOx (+)	5.58	1/42	0.023	0.117
Estimated marginal means (SE)						
Variables		CAP		CAP	Hypertension	YES
		NO CAP			NO	
WHO		97.7 (4.1)		82.7 (2.5)	NS	NS
BDI		2.3 (3.1)		13.5 (1.9)	NS	NS
HDRS		3.6 (1.7)		11.5 (1.0)	5.5 (1.1)	9.5 (1.5)

HDRS Hamilton Depression Rating Scale, *BDI* Beck Depression Inventory, *WHO* WHO Quality of Life-BREF; *NOx* nitric oxide metabolites

Discussion

The first major finding of this study is that subjects with CAP and depression show highly increased root canal endotoxin levels as compared to subjects with CAP without depression and normal controls. In addition, there was a very strong positive association between CAP or root canal endotoxin levels and severity of depression as measured using the HDRS

Table 5 Results of multivariate GLM analyses with the three Hamilton Depression Rating Scale (HDRS) subscale scores as dependent variables and chronic apical periodontitis (CAP), root canal endotoxin levels, or oxidative and nitrosative stress biomarkers as explanatory variables

Tests	Dependent variables	Explanatory variables	*F*	df	*p* values	Partial eta squared
Multivariate #1	HDRSd, HDRSv, HDRSps	CAP	6.23	3/43	0.001	0.303
Between-subject effects	HDRSd	CAP (+)	6.42	1/45	0.015	0.125
	HDRSv	CAP (+)	18.53	1/45	<0.001	0.292
	HDRSps	CAP (+)	9.73	1/45	0.003	0.178
Multivariate #2	HDRSd, HDRSv, HDRSps	Endotoxin	58.58	3/43	<0.001	0.641
Between-subject effects	HDRSd	Endotoxin (+)	21.14	1/45	<0.001	0.320
	HDRSv	Endotoxin (+)	62.83	1/45	<0.001	0.583
	HDRSps	Endotoxin (+)	32.94	1/45	<0.001	0.423
Multivariate #3	HDRSd, HDRSv, HDRSps	NOx	2.97	3/39	0.043	0.186
		TRAP	2.96	3/39	0.044	0.185
Between-subject effects	HDRSd	NOX (+)	7.41	1/41	0.009	0.153
	HDRSv	TRAP (+)	7.48	1/41	0.009	0.154
	HDRSps	TRAP (+)	6.51	1/41	0.015	0.137

HDRSd HDRS subscore "core depressive symptoms," that is sum of items 1, 2, 3, 7, and 17; *HDRSv* HDRS subscore "vegetative symptoms," that is sum of items 4, 5, 6, 8, and 16; *HDRSps* HDRS subscore "physio-somatic symptoms," that is sum of items 11, 12, 13, 14, and 15; *NOx* nitric oxide metabolites; *TRAP* total radical trapping antioxidant parameter

Table 6 Results of multivariate GLM analyses with the four WHO Quality of Life (QoL)-BREF domains (physiological, psychological, social relationships, and environment) as dependent variables and chronic apical periodontitis (CAP) or root canal endotoxin as explanatory variables

Tests	Dependent variables	Explanatory variables	*F*	df	*p* value	Partial eta squared
Multivariate #1	Physical, psychological, social, environment	CAP	3.21	4/41	0.022	0.238
Between-subject effects	Psychological	CAP (−)	8.12	1/44	0.007	0.156
	Social	CAP (−)	11.22	1/44	0.002	0.203
	Environment	CAP (−)	8.61	1/44	0.005	0.164
Multivariate #2	Physical, psychological, social, environment	Endotoxin	3.84	4/41	0.010	0.273
Between-subject effects	Physical	Endotoxin (−)	10.48	1/44	0.002	0.192
	Psychological	Endotoxin (−)	13.05	1/44	0.001	0.229
	Social	Endotoxin (−)	9.07	1/44	0.004	0.171
	Environment	Endotoxin(−)	8.48	1/44	0.006	0.162
Estimated marginal means (SE)						
Variables			CAP			
			NO CAP		CAP	
Psychological			26.2 (1.4)		21.7 (0.8)	
Environment			12.8 (0.6)		10.5 (0.3)	
Social			29.3 (1.4)		24.6 (0.8)	

(psychiatric interview) and BDI (self-rating). The link between periodontitis and depression is important as periodontal disease is a serious public health problem with an estimated prevalence of 47% [49]. The current findings extend previous reports that dental health and periodontitis may be related to depression. At the population level, a number of studies show a significant association between periodontitis and depression [27, 28]. Psychiatric patients may have poor oral health status [50], which could aggravate dental care and thus periodontal disease and periodontitis. In addition, patients with clinical depression are more likely to have tooth loss and less likely to use oral health services [51]. However, not all studies support this association, including a study in an elderly population, which found no significant association between depression and measures of oral health, including periodontal disease [52].

Our study shows that the association between depression and CAP is attributable, at least in part, to increased root canal endotoxin levels in periodontitis patients. It is known that the bacteria associated with periodontal disease are predominantly Gram-negative bacteria, including *P. gingivalis* [22, 32]. CAP and chronically increased endotoxin levels in root canals, may play a pathophysiological role in the onset of depression. Preclinical models show that a state of chronic depression may be induced by chronic elevations in endotoxin, using repeated-intermittent endotoxin administration for 4 months [53]. This is further corroborated by previous studies showing that the endotoxin load produced by Gram-negative commensal gut bacteria is increased in depression, especially chronic depression [15, 16, 20]. The Gram-negative bacteria that play a role in gut-brain axis-linked depression are *H. alvei*, *P. aeruginosa*, *M. morganii, P. mirabilis*, *P. putida*, *C. koseri*, and *K. pneumoniae* [19, 20], while the most important Gram-negative bacteria related to periodontitis are *P. gingivalis*, *P. intermedia*, *F. nucleatum*, *P. aeruginosa*, *B. forsythus*, *C. rectus*, and *Treponema* sp. [22, 32, 33]. A study is underway to determine changes in the microbiome in CAP-related depression (Gomes et al.). Some possibilities are (a) increased endotoxin of *P. aeruginosa* is a common denominator linking depression to "leaky gut" and "leaky teeth"; (b) the endotoxin of *P. gingivalis* is a major determinant of depression, as this bacteria is a major player in CAP [22]; and (c) perhaps most plausible is a general increase in endotoxins through leaky teeth.

CAP is an inflammatory condition whereby accumulation of bacteria in dental plaque biofilms causes lesions in tooth-supporting tissues, including gum, connective, and periodontal tissues and eventually alveolar bone [24, 54]. Gram-negative bacteria are keystone periodontal pathogens related to CAP [22]. Accordingly, we found significantly increased root canal endotoxin levels in patients with CAP as compared to healthy controls without periodontitis. The link between CAP and increased root canal endotoxin levels may explain previous findings that inflammation in periodontal disease is mediated by M1 macrophagic pro-inflammatory cytokines [55, 56]. Increased endotoxin levels activate TLR 2/4 thereby increasing inflammatory responses leading to M1 macrophagic activation [17].

The second major finding of this study is that increased root canal endotoxin levels were strongly associated with enhanced indicants of systemic O&NS, namely an elevated O&NS index as well as raised levels of AOPP and NOx.

This indicates that elevated endotoxins in CAP may lead to systemic responses, including increased NOx production and oxidation of proteins. These findings extent previous data showing that periodontitis is accompanied by systemic inflammatory responses, as exemplified by increased serum levels of CRP [25]. Importantly, we detected that depression was accompanied by increases in the same O&NS biomarkers. Therefore, increased root canal endotoxin in CAP may be another pathway linking Gram-negative bacteria with systemic O&NS processes and mood symptoms. Clinical depression is frequently accompanied by activated O&NS pathways some of which are relevant to the current study, namely increased levels of peroxides [2, 10, 57], lipid peroxidation [2, 10, 13, 57–60], oxidation of proteins [2, 10, 60], and increased nitrosylation or production of NO [2, 14, 61, 62]. Previously, we have described many mechanisms whereby activated O&NS pathways may cause neuronal dysfunctions, including increased neurotoxicity and cytotoxicity, neurodegeneration, lowered neurogenesis, neuroplasticity, and neurotrophic expression, collectively referred to as neuroprogression [63]. For example, increased AOPP may induce inflammatory responses, monocytic activation, advanced glycation end products (AGE), and a receptor for AGE (RAGE), while AOPP are potent high-density lipoprotein receptor antagonists [64–66]. Increased NOx may lead to hypernitrosylation, which may induce neurodegenerative responses and neuronal dysfunctions [67].

The third major finding of this study is that root canal endotoxin levels were most strongly associated with the vegetative and physio-somatic components of the HDRS. Previously, we have shown that a raised bacterial load, originating from gut commensal bacteria, is related to chronic fatigue syndrome (CFS), a neuroimmune disorder characterized by physio-somatic symptoms [68]. In CFS, increased LPS load is associated with neurocognitive deficits, abdominal discomfort, muscular tension, and fatigue [69, 70], and with increases in lipid peroxidation, hypernitrosylation [71], and inflammatory responses [70].

In contrast to the a priori hypothesis, we found that TRAP, an index of total antioxidant capacity, was strongly and positively associated with depression, CAP, and increased root canal endotoxin levels. As an index of antioxidant defenses, it was expected that lowered TRAP values would be evident in depression, which is accompanied by activated O&NS pathways and lowered antioxidant defenses (10, 11). Chang et al. [72] reported that depressed patients had significantly lower TRAP levels, which were additionally inversely correlated with the HDRS score and superoxide radical levels. Also, subjects with a history of suicide attempts show decreased TRAP levels [73]. In addition, lowered levels of other antioxidants and antioxidant enzymes are frequently found in depression, including lowered zinc, coenzyme Q10, vitamins E and C, HDL-cholesterol, albumin, tryptophan and tyrosine, glutathione, glutathione peroxidase, and catalase [2, 6, 10, 60, 74]. Nevertheless, the very strong associations between TRAP, on the one hand, and depression and endotoxin, on the other, may suggest a highly specific response of TRAP to endotoxin in CAP patients. It is possible that elevated endotoxin increases xanthine oxidase activity [75, 76], which in turn increases uric acid [77], an important component of TRAP [78]. Interestingly, at lower concentrations uric acid is an antioxidant, although priming TLR-induced cytokine production, while at higher concentrations uric acid may induce ROS and inflammation [79, 80]. Unfortunately, we did not assay uric acid in this study. Unexpectedly, PON1 total activity is not decreased in depression and is not related to increased endotoxin levels. Indeed, serum PON1 activity is lower in major depression in some [81], but not all [82] studies. A challenge with LPS (intravenously) may reduce PON1 serum activity and induce an inflammatory response [83]. Although we found lowered –SH groups in subtypes of depression, such as prenatal depression (Roomruangwong et al., submitted), the current study was unable to find any changes in –SH groups related to depression, chronic periodontitis, or increased endotoxin.

Importantly, we found that quality of life was significantly lowered in CAP participants and those with elevated root canal endotoxin levels. Such effects may be related to effects of endotoxin inducing O&NS pathways and inflammation and thus depression or to multiple detrimental effects of O&NS on the cardiovascular system, including AOPP accelerating arteriosclerosis [11]. Indeed, periodontal disease and periodontitis may predict inflammatory disorders, such as cardiovascular disease and increased mortality in coronary artery disease and diabetes [26, 84, 85]. Furthermore, periodontitis may induce leaky gut [86] thereby aggravating immune-inflammatory and O&NS responses as well as inducing autoimmune pathways [16], which may further lower quality of life. These findings may be interpreted to indicate that previous reports on relationships between periodontal disease and low self-esteem, feelings of loneliness and elevated psychological stress [29–31] may be attributed to the effects of endotoxin inducing immune-inflammatory and O&NS pathways and thus depression and stress symptoms. Nevertheless, O'Neil et al. [87] found that the positive association between poor dental health and depression was independent of CRP levels [87]. Future research should examine the associations between CAP, LPS and more adequate inflammatory markers, including serum levels of interleukin-6 and haptoglobin, and intracellular signaling networks, including nuclear factor κB.

This study has some strengths and limitations that should be discussed when interpreting the results. Firstly, this is a case-control study and therefore, no inferences can be made on causality. Secondly, it would have been interesting if we would have assayed uric acid and xanthine oxidase to interpret the effects of endotoxin on TRAP values. It could be argued that some trend-level findings, namely effects of hypertension

on severity of depression, should be reported as negative. Nevertheless, a mild relationship between hypertension and depression was reported in a recent meta-analysis [88]. In addition, we used background predictors (not only hypertension, but also age and sex) in order to minimize the variance in the data with the aim to delineate more precisely the relationships between the primary dependent (including severity of illness) and independent (including CAP and LPS) variables. Strengths are that we used multivariate analyses to adjust for different background variables and that univariate associations were interpreted only when there were significant multivariate effects, thereby reducing type I errors. The present results require replication, as well as clarification as to interpretation, given that a recent meta-analysis of CAP and depression concluded that, although suggestive, no firm association can made on the basis of data to date [89].

All in all, elevated levels of root canal LPS in CAP are associated with depression and a lowered quality of life, which may be partly explained by activated O&NS pathways, especially increased NOx and associated hypernitrosylation. Improving dental health, and thereby decreasing CAP, may be especially important in depressed patients, including by attenuating the immune-inflammatory and O&NS pathways that can underpin depression. This should decrease the two-way interactions of depression and CAP, while enhancing quality of life.

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Author Contributions CG, MM, HOV, and SOVN made the design of the study. Participants were recruited and screened by CG. Biomarker assays were performed by FCM, DSB, LSA, HCCP, THLB, and NRM. MM performed the statistical analyses. All authors contributed equally to the writing of the paper. All authors agreed upon the final version of the paper.

Compliance with Ethical Standards

Conflict of Interest The authors have no conflict of interest with any commercial or other association in connection with the submitted article.

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