



Clinical Investigation of Matrix Metalloproteinases, Tissue Inhibitors of Matrix Metalloproteinases, and Matrix Metalloproteinase/Tissue Inhibitors of Matrix Metalloproteinase Complexes and Their Networks in Apical Periodontitis

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Abstract

Introduction: This clinical study investigated the levels of metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) and respective forms (MMP/TIMP complexes) in apical periodontitis to determine their networks in the development of clinical/radiographic features, thus quantifying the levels of endotoxins (lipopolysaccharides) present in primarily infected root canals with apical periodontitis. **Methods:** Twenty primarily infected root canals with apical periodontitis were selected. The presence of pain on palpation, tenderness to percussion, and the size of the radiographic lesion were recorded. The levels of MMPs (MMP-1, -2, and -9), TIMPs (TIMP-1 and -2), and their MMP/TIMP complexes (MMP-1/TIMP-1, MMP-1/TIMP-2, MMP-2/TIMP-1, MMP-2/TIMP-2, MMP9/TIMP-1, and MMP-9/TIMP-2) present in the periapical interstitial fluid were measured using the enzyme-linked immunosorbent assay. The kinetic chromogenic LAL test was used to quantify endotoxins. **Results:** A higher mean level of MMP-9 (968.35 ± 342.00 pg/mL) was followed by MMP-2 (894.00 ± 591.62 pg/mL) and MMP-1 (789.43 ± 342.83 pg/mL). The linear regression analysis revealed a positive association of MMP-1 with both MMP-2 and MMP-9 (all $P < .001$). TIMP-1 (481.79 ± 86.09 pg/mL) (24/24) was found in higher levels than TIMP-2 (206.45 ± 86.09 pg/mL) ($P < .05$), including a positive correlation of MMP-1 with both TIMP-1 and TIMP-2 (all $P < .05$). Higher mean levels of MMP1, -2, and -9

were found in teeth with larger-size radiolucent lesions (>7 mm) compared with smaller ones (≤ 7 mm) (all $P < .01$). Higher levels of MMP-1 decreased the chance of TTP, whereas MMP-9 (odds ratio = 0.97) increased the chance of pain on percussion (odds ratio = 1.01). Higher levels of endotoxins present in root canals were positively correlated with larger amounts of MMP-9 ($P < .05$). **Conclusions:** MMPs, TIMPs, and their complexes (MMP/TIMP) are involved in apical periodontitis by interacting with complex networks in the development of clinical features and the severity of bone destruction. (*J Endod* 2016;42:1082–1088)

Key Words

Endodontics, endotoxin, infection, root canal

Apical periodontitis is a tissue breakdown established in the periapical tissue provoked by the interplay between bac-

terial infection of the root canal systems and the local immune response (1). Such a local immune response is very complex and involves degradation of the extracellular matrix (ECM) components, the main components of connective tissue (2).

Matrix metalloproteinases (MMPs) (3), a family of zinc- and calcium-dependent endopeptidases, are strongly associated with the breakdown of ECM (4) and play a major role in bone remodeling and resorption (5). According to their target protein, MMPs are divided into several families (6) as follows: collagenase (MMP-1 (7)), gelatinases (MMP-2 (8)), stromelysin (MMP-3 (7)), and matrixin (MMP-9 (9)). Different subtypes of MMPs, including MMP-1, -2, -3, -7, -8, and -9, have been detected in apical periodontitis (3,10–16). Of a clinical concern, elevated MMP levels have been correlated with nonhealing lesions (17, 18).

Significance

MMPs, TIMPs, and their complexes play a role in apical periodontitis

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The zinc-dependent endopeptidase activities of the MMPs are inhibited specifically by their intrinsic inhibitors called tissue inhibitors of MMPs (TIMPs) (19–21). TIMPs have been detected in apical periodontitis (16, 22), with TIMP-1 and -2 being multifunction proteins (23). Particularly, TIMP-1 has been suggested as an enhancer or inhibitor of bone resorption depending on whether TIMP-1 concentrations are low or high, respectively (24). Despite the biological activities of MMPs, independent of the MMP-inhibitory activity, the imbalance between the levels of MMPs and TIMPs is critical for ECM remodeling being able to provoke tissue destruction (23). Thus, TIMPs can bind tightly to the active proteinases (MMPs) to form the MMP/TIMP complex (25–27). The MMP/TIMP complex has been speculated by other research fields as a potential biological biomarker for the possible prediction of changes in the course of diseases (28–30).

To help understand the participation of MMPs and TIMPs and their network in apical periodontitis, this clinical study sought to investigate the levels of MMPs and TIMPs and their respective forms (MMP/TIMP complexes) by determining their networks in the development of clinical/radiographic features, thus quantifying the levels of endotoxins (lipopolysaccharides [LPSs]) present in primarily infected root canals with apical periodontitis.

Materials and Methods

Patient Selection

Twenty patients attending São José dos Campos Dental School, São José dos Campos, São Paulo, Brazil, for primary endodontic treatment were included in the present study. The age of patients ranged from 22–45 years old (mean = 35 years, 7 women and 13 men). A detailed dental and general history was obtained from each patient. Those who had received antibiotic treatment during the past 3 months and/or who had any systemic health disorder that might affect the inflammatory response were excluded.

The Human Research Ethics Committee of the São José dos Campos Dental School approved the protocol describing the sample collection for this investigation, and all the volunteer patients signed an informed consent form.

All the single-rooted teeth were maxillary teeth with primary endodontic infection showing the presence of 1 root canal and the absence of periodontal pockets deeper than 4 mm. Teeth that could not be isolated with a rubber dam were also excluded. None of them reported any type of systemic disease. The following clinical/radiographic features were recorded: clinical symptomatology (spontaneous pain) (11/20), pain on palpation (9/20), tenderness to percussion (7/20), and size of radiolucent area >7 mm (11/20).

Sampling Procedures

The files, instruments, and all materials used in this study were treated with Co⁶⁰ gamma radiation (20 kGy for 6 hours) for sterilization and elimination of pre-existing endotoxins (EMBRARAD; Empresa Brasileira de Radiação, Cotia, SP, Brazil) (31). The method used for disinfection of the operative field has been previously described elsewhere (32). Briefly, the teeth were isolated with a rubber dam. The crown and surrounding structures were disinfected with 30% H₂O₂ (volume/volume) for 30 seconds followed by 2.5% sodium hypochlorite (NaOCl) for the same period of time and then inactivated with 5% sodium thiosulfate. The sterility of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it onto blood agar plates, which were then incubated both aerobically and anaerobically.

A 2-stage access cavity preparation was made without the use of water spray but under manual irrigation with sterile/apyrogenic saline solution and by using a sterile/apyrogenic high-speed diamond bur. The first stage was performed to promote a major removal of contaminants, including carious lesion and restoration. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the protocol described previously. Sterility of the internal surface of the access cavity was checked as previously described, and all procedures were performed aseptically. An endotoxin sample was taken by introducing sterile/apyrogenic paper points (size #15; Dentsply Maillefer, Ballaigues, Switzerland) into the full length of the canal determined radiographically and retained in position for 60 seconds for sampling. Immediately after, the sample was placed in pyrogen-free glass and immediately suspended in 1 mL limulus amoebocyte lysate water and frozen at –80°C for further dosage of endotoxins using a kinetic chromogenic limulus amoebocyte lysate assay (Lonza, Walkersville, MD).

After endotoxin sampling, the root canal length was determined from the preoperative radiograph and confirmed using an apex locator (RomiApex A-15; Romidan Dental Solution, Kiryat-Ono, Israel). The root canals were prepared by using Mtwo files (VDW, Munich, Germany) according to the manufacturer's instructions. The files were adapted to an electric motor (VDW), and all instruments were used within the working length in a gentle in-and-out motion. The instrumentation sequence was as follows: 0.04 taper size #10 instrument, 0.05 taper size #15 instrument, 0.06 taper size #20 instrument, 0.06 taper size #25 instrument, 0.05 taper size #30 instrument, 0.04 taper size #35 instrument, 0.04 taper size #40 instrument, and 0.07 taper size #25 instrument. The use of each instrument was followed by irrigation with disposable syringes and 30-G NaviTip needles (Ultra-dent, South Jordan, UT) using 5 mL 2.5% NaOCl solution. Before the second sampling after instrumentation, NaOCl was inactivated with 5 mL sterile 0.5% sodium thiosulfate during a 1-minute period, which was then removed with 5 mL sterile/apyrogenic water. Next, the second bacterial sampling was performed as previously described.

After root canal instrumentation, the samples were collected from the interstitial fluid in the apical periodontitis according to Martinho et al (31). Three sterile paper points were introduced into the root canal through the root apex (2 mm) and kept there for 1 minute for sampling inflammatory contents from the interstitial fluid in the apical tissues. After withdrawal, the paper points were cut 4 mm from the tip, dropped into a 1.5-mL sterile plastic tube, and immediately stored at –80°C for further analysis of the cytokines.

Quantification of MMPs, TIMPs, and MMP/TIMP Complexes

Quantification of Inflammatory Cytokines (Enzyme-linked Immunosorbent Assay)

The amount of MMPs (MMP-1 [Cat #DY901], -2 [Cat #DY902], and -9 [Cat #DY911]), TIMPs (TIMP-1 [Cat #DY970] and -2 [Cat #DY971]), and MMP/TIMP complexes (MMP-1/TIMP-1 [Cat #DY1550], MMP-1/TIMP-2 [Cat #DY1553], MMP-2/TIMP-1 [Cat #DY1496], MMP-2/TIMP-2 [Cat #DY1497], MMP-9/TIMP-1 [Cat #DY1449], and MMP-9/TIMP-2 [Cat #DY1453]) sampled from the interstitial fluid in the apical tissues were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc, Minneapolis, MN). Next, standard and sample solutions were added to the ELISA well plate, which had been precoated with specific monoclonal capture antibody for MMP-1, MMP-2, MMP-3, TIMP-1, TIMP-2, MMP-1/TIMP-1, MMP-1/TIMP-2, MMP-2/TIMP-1, MMP-2/TIMP-2, MMP-9/TIMP-1, and MMP-9/TIMP-2. After it was gently shaken for 3 hours at room temperature, the polyclonal anti-MMP-1, –MMP-2, –MMP-3, –TIMP-1, –TIMP-2, –MMP-1/TIMP-1,

–MMP-1/TIMP-2, –MMP-2/TIMP-1, –MMP-2/TIMP-2, –MMP-9/TIMP-1, and –MMP-9/TIMP-2 antibodies conjugated with horseradish peroxidase were added to the solution and incubated for 1 hour at room temperature. A substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react for 20 minutes. The levels of MMPs, TIMPs, and MMP/TIMP complexes were assessed with a micro-ELISA reader at 450 nm and normalized with standard solution. Each densitometric value, expressed as mean \pm standard deviation, was obtained from 3 independent experiments.

Quantification of Endotoxins

Quantification of Endotoxins (LPSS) by Kinetic Chromogenic Limulus Amebocyte Lysate Assay. The kinetic chromogenic limulus amebocyte lysate assay (Lonza) was used for quantification of endotoxins. *Escherichia coli* endotoxin was used as the standard. A positive control (root canal sample contaminated with a known amount of endotoxin) was included for each sample to determine the presence or absence of interfering agents. For the test, 100 μ L apyrogenic water (reaction blank), 5 standard endotoxin solutions (0.005–50 endotoxin units [EU]/mL), root canal samples, and positive controls (each root canal sample contaminated with a known concentration of endotoxin [10 EU/mL]) were added to a 96-well apyrogenic plate. The tests were performed in quadruplicate. The plate was incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 10 minutes in a kinetic-QCL reader, which was coupled to a microcomputer by means of the WinQCL software. Next, 100 μ L chromogenic reagent was added to each well. After the beginning of the kinetic test, the software continuously monitored absorbance at 405 nm in each microplate well and automatically calculated the log/log linear correlation between the reaction time of each standard solution and the corresponding endotoxin concentration.

Statistical Analysis

Data collected for each case were tabulated in duplicate into a spreadsheet and statistically analyzed using STATA 12.0 software (StataCorp, College Station, TX). Initially, a descriptive analysis using the

Bartlett test was performed in order to explore data distribution. Data presenting non-normal distribution were appropriately transformed. Next, 1-way analyses of variances were conducted to determine the concentrations of MMPs, TIMPs, and MMP/TIMP complexes according to clinical and radiographic features. The Pearson correlation test was performed to correlate the levels of MMPs, TIMPs, and the MMP/TIMP complex. Linear regression was used to determine the possible associations of MMPs, TIMPs, and their complex (MMP/TIMP) with the size of the radiolucent area (mm). Multivariate logistic regression models were performed to test associations between clinical features and MMPs. For variable selection in the multivariate logistic regression, the stepwise backward approach was used. Variables with a P value $< .20$ in the crude analysis were included in the adjusted model as confounders. The significance level was set as 5% in the final model after adjustment.

Results

Quantification of MMPs (MMP-1, -2, and -9), TIMPs (TIMP-1 and -2), and Complexes (MMPs/TIMPs) and Endotoxin

MMP-1, -2, and -9 were detected in 100% (20/20) of the samples. Higher mean levels of MMP-9 (968.35 ± 342.00 pg/mL) followed by MMP-2 (894.00 ± 591.62 pg/mL) and MMP-1 (789.43 ± 342.83 pg/mL) were recorded. The linear regression analysis revealed a positive association of MMP-1 with MMP-2 ($\beta = 0.56$, $R^2 = 0.952$, $P < .001$) and MMP-9 ($\beta = 0.57$, $R^2 = 0.889$, $P < .001$). Higher mean levels of TIMP-1 (481.79 ± 86.09) (20/20) were found compared with TIMP-2 (206.45 ± 86.09 pg/mL) (20/20) ($P < .05$). The linear regression analysis revealed positive associations of MMP-1 with TIMP-1 ($\beta = 0.78$, $R^2 = 0.212$, $P < .05$) and TIMP-2 ($\beta = 1.83$, $R^2 = 0.231$, $P < .05$). All MMP/TIMP complexes (MMP-1/TIMP1, MMP-1/TIMP-2, MMP-2/TIMP-1, MMP-2/TIMP-2, MMP-9/TIMP-1, and MMP-9/TIMP-2) were detected in 100% of the samples (20/20). Individual mean values of the MMP/TIMP complex are shown in Figure 1. MMP-2/TIMP-2 (394.54 ± 266.29 pg/mL) and MMP-9/TIMP-1 (546.56 ± 388.04 pg/mL) were encountered in higher levels compared with all other complexes tested. Endotoxins were detected in

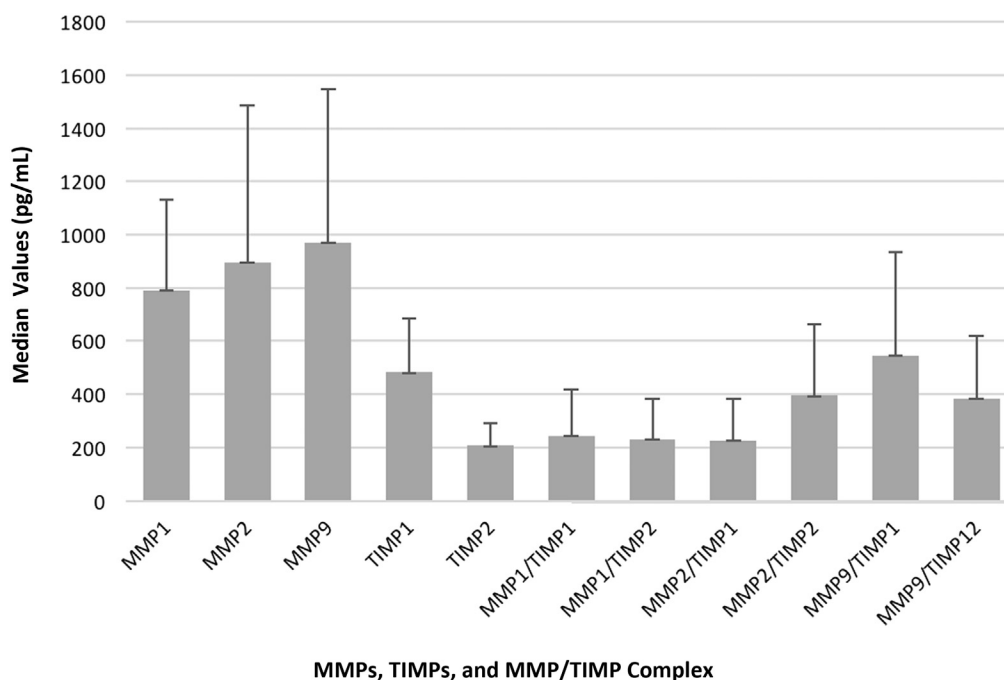


Figure 1. Distribution of the median values (pg/mL) of MMPs, TIMPs, and the MMP/TIMP complex encountered in apical periodontitis.

TABLE 1. Concentration of MMP, TIMP, and the MMP/TIMP Complex According to Clinical Symptoms and Radiographic Findings

	Lesion size (mm)			POP			Symptom		
	<7	>7	P value	Absence	Presence	P value	Absence	Presence	P value
MMP-1	536.99 (239.84)	1041.88 (221.33)	<.0001	718.55 (333.10)	1072.99 (235.82)	.060	719.73 (275.41)	893.98 (423.10)	.277
MMP-2	430.57 (260.46)	1356.75 (441.03)	<.0001	773.22 (565.67)	1375.43 (479.57)	.060	735.66 (480.60)	1030.66 (692.90)	.148
MMP-9	547.04 (241.68)	1389.66 (505.59)	<.0001	827.77 (512.60)	1530.65 (533.58)	.020	763.53 (364.97)	1275.57 (722.35)	.049
Total MMP	1513.8 (739.58)	3788.24 (1138.53)	<.0001	2319.05 (1390.27)	3978.92 (1244.41)	.043	2218.18 (1099.92)	3300.27 (1833.27)	.114
TIMP-1	521.79 (252.97)	441.70 (132.82)	.387	456.15 (231.83)	584.14 (97.47)	.265	478.82 (228.94)	486.13 (164.74)	.939
TIMP-2	223.62 (108.41)	189.30 (56.92)	.382	195.49 (91.64)	250.34 (41.77)	.256	205.21 (98.12)	208.34 (70.60)	.929
Total TIMP	745.42 (361.39)	631.01 (189.74)	.392	651.64 (305.48)	834.49 (139.25)	.255	684.04 (327.06)	694.48 (235.35)	.932
MMP-1/TIMP-1	413.21 (14.97)	75.28 (7.35)	<.0001	264.65 (172.82)	162.64 (175.31)	.306	273.39 (176.68)	200.53 (170.98)	.372
MMP-1/TIMP-2	377.32 (18.88)	84.42 (10.21)	<.0001	249.80 (149.68)	155.18 (150.99)	.273	258.41 (154.81)	189.55 (144.77)	.331
MMP-2/TIMP-1	376.11 (32.84)	72.71 (7.81)	<.0001	241.38 (155.97)	156.53 (165.88)	.348	244.82 (156.10)	193.79 (164.72)	.492
MMP-2/TIMP-2	641.77 (116.59)	147.52 (20.01)	<.0001	424.03 (262.94)	277.10 (283.58)	.337	419.33 (247.84)	357.62 (305.52)	.624
MMP-9/TIMP-1	904.19 (47.73)	150.63 (11.83)	<.0001	576.66 (388.58)	330.38 (366.08)	.267	585.45 (380.98)	440.35 (407.66)	.427
MMP-9/TIMP-2	611.06 (19.87)	155.76 (15.42)	<.0001	411.86 (235.60)	269.61 (219.67)	.289	418.10 (235.80)	331.39 (237.40)	.432

	Previous Pain			TTP		
	Absence	Presence	P value	Absence	Presence	P value
MMP-1	838.59 (265.50)	641.97 (524.76)	.278	779.79 (290.80)	818.36 (510.86)	.834
MMP-2	951.74 (527.07)	719.40 (799.77)	.462	843.85 (522.06)	1043.09 (819.41)	.529
MMP-9	997.23 (499.68)	879.63 (840.68)	.703	893.93 (476.63)	1191.61 (846.09)	.330
Total MMP	2767.66 (1270.38)	2241.09 (2164.49)	.493	2516.94 (1266.46)	3053.27 (2174.98)	.502
TIMP-1	523.79 (198.10)	355.62 (166.83)	.106	501.70 (213.05)	421.88 (163.88)	.456
TIMP-2	224.48 (84.90)	152.41 (71.50)	.109	215.01 (91.31)	180.81 (70.24)	.557
Total TIMP	748.28 (283.00)	508.02 (238.32)	.205	716.22 (304.36)	602.70 (234.11)	.478
MMP-1/TIMP-1	235.63 (178.35)	270.09 (175.86)	.712	257.22 (177.40)	205.34 (175.25)	.577
MMP-1/TIMP-2	222.14 (153.77)	257.06 (156.10)	.664	242.62 (155.82)	195.62 (145.68)	.561
MMP-2/TIMP-1	210.45 (156.10)	266.29 (171.63)	.507	231.93 (158.96)	201.85 (168.33)	.722
MMP-2/TIMP-2	360.54 (246.99)	496.95 (325.39)	.334	400.62 (254.88)	372.82 (330.05)	.867
MMP-9/TIMP-1	492.71 (376.16)	631.49 (449.41)	.503	547.81 (380.78)	466.19 (488.90)	.695
MMP-9/TIMP-2	367.42 (236.85)	431.40 (245.68)	.610	395.48 (234.81)	347.19 (255.69)	.700

MMP, matrix metalloproteinase; POP, pain on percussion; TIMP, tissue inhibitor of MMP.

Bold numbers signify $P < .05$ (statistical significance).

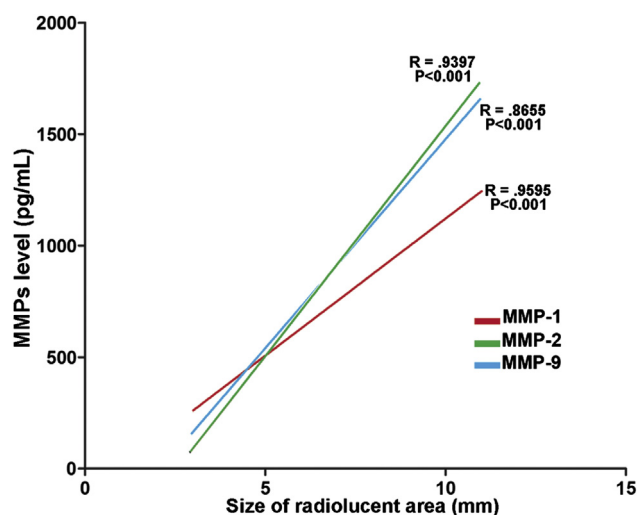


Figure 2. The Pearson correlation between MMP-1, -2, and -9 and the size of the radiolucent area.

100% of the root canal samples (20/20), with mean values of 10.92 EU/mL (1.75–128 EU/mL). Higher levels of endotoxins present in root canals were positively correlated with larger amounts of MMP-9 ($P < .05$).

MMPs Versus Clinical/Radiographic Features

Higher mean levels of MMP-1 (1041.88 ± 221.33 pg/mL vs 536.99 ± 239.84 pg/mL, $P < .001$), MMP-2 (1356.75 ± 441.03 pg/mL vs 430.57 ± 260.46 pg/mL, $P < .001$), and MMP-9 (1389.66 ± 505.59 pg/mL vs 547.04 ± 241.68 pg/mL, $P < .001$) were found in teeth with larger-size radiolucent lesions (>7 mm) compared with smaller ones (≤ 7 mm), respectively (Table 1). A positive correlation between higher levels of MMP-1, -2, and -9 and a larger area of bone destruction (Fig. 2) was also observed. Higher levels of MMP-2

TABLE 2. Multivariate Logistic Regression Models Performed to Test Associations between Clinical Features and Matrix Metalloproteinase (MMP)

Clinical features	Adjusted odds ratio	95% confidence interval		P value
		Lower limit	Upper limit	
Pain*				
MMP-1	0.98	0.96	0.99	0.046
MMP-2	—	—	—	—
MMP-9	1.01	1.00	1.03	0.049
Tenderness to percussion†				
MMP-1	0.97	0.95	0.99	0.047
MMP-2	1.01	1.00	1.04	0.050
MMP-9	—	—	—	—
Pain on palpation‡				
MMP-1	—	—	—	—
MMP-2	—	—	—	—
MMP-9	1.01	1.00	1.02	0.040
Any symptom‡				
MMP-1	0.98	0.95	0.99	0.037
MMP-2	—	—	—	—
MMP-9	1.03	1.01	1.05	0.046

*Adjusted for tissue inhibitor of matrix metalloproteinase (TIMP)-2.

†Adjusted for TIMP-1, TIMP-2, and interleukin (IL)-1 β .

‡Adjusted for IL-6.

decreased the odds of clinical symptomatology (odds ratio [OR] = 0.98), whereas higher levels of MMP-9 increased this chance (OR = 1.01) (Table 2). In relation to tenderness to percussion, higher levels of MMP-1 reduced by 3% the chance of this outcome (OR = 0.97), whereas higher levels of MMP-2 increased by 1% (OR = 1.01). Thus, higher levels of MMP-9 increased the chance of pain on palpation (OR = 1.01) (Table 2).

Discussion

The data obtained in the present study revealed that MMPs, TIMPs, and their complexes (MMP/TIMP) are involved in apical periodontitis because they interact with complex networks in the development of clinical features and the severity of bone destruction.

All apical periodontitis samples were found to be positive for MMP-1, -2, and -9, which supports their role in the establishment/development of the periapical inflammatory process (3,11,14–16). Higher mean levels of MMP-9 and MMP-2 were recorded. Increased levels of MMP-2 and -9 have been reported in teeth with apical periodontitis and in wall extracts from jaw cysts (3, 10). Both gelatinases, gelatinase A (MMP-2) and B (MMP-9), are synthesized by both fibroblasts and pulp cells, and their presence favors the degradation of type IV collagens/gelatins (main components of the basement membrane) as well as denatured gelatins, lamins, elastins, fibronectins, and basement membrane zone-associated components (3). Additionally, increased levels of MMP-2 have been associated with the presence of *Porphyromonas endodontalis* and *P. gingivalis*, which are commonly found in primary infection (32, 33), as analyzed in the present study.

The linear regression analysis has revealed a positive correlation of MMP-1 with MMP-2 (beta = 0.56, $R^2 = 0.952$, $P < .001$) and MMP-9 (beta = 0.57, $R^2 = 0.889$, $P < .001$), thus elucidating their association with apical periodontitis. Based on the R^2 values from this analysis, it is not unreasonable to assume that MMP-1 had a significant implication for the production of MMP-2 and MMP-9 in 95.2% and 88.9% of the cases, respectively. Of a clinical relevance, higher levels of MMP-1, -2, and -9 were found in teeth with a larger area of bone destruction. Such a finding is in accordance with the literature regarding the assumption that MMPs are involved in jaw cyst expansion and larger areas of bone destruction (34, 35). Indeed, Delaissé et al (35) reported that MMP-9 is a cooperative enzyme that participates in the activation-resorption and formation cycle involved in both bone remodeling and pathological bone destruction.

In this study, the multivariate logistic regression model elucidated the association between MMPs and the presence of clinical features (13, 15, 16). Higher levels of MMP-2 decreased the chance of clinical symptoms, whereas higher levels of MMP-9 increased it. It is worth pointing out that the gelatinases (MMP-2 and -9) appear to regulate neuropathic pain after peripheral nerve injury through degenerative and proinflammatory mechanisms (36). Additionally, MMP-9 expression also appears to mediate pain behavior (15, 37). In relation to tenderness to percussion, higher levels of MMP-1 reduced the chance of this outcome, whereas higher levels of MMP-2 increased it. Thus, higher levels of MMP-9 increased the chance of pain on palpation. It should be also highlighted that both tenderness to percussion and pain on palpation are clinical parameters indicative of an inflammatory disease in the periapical tissues.

It is known that MMPs are specifically inhibited by their intrinsic inhibitors, the so-called tissue inhibitors of MMP (TIMPs) (20). TIMP-1 and -2 are well characterized and play a multifunctional role as proteins (23). In this study, TIMP-1 and -2 were found in all apical periodontitis, reinforcing their role in the pathogenesis of inflammatory periapical

lesions (16, 22). This result corroborates previous findings showing that the expression of TIMP-1 was also significantly increased in the chronic stages of such lesions (11, 22).

Although the MMP's biological activities are independent of the MMP-inhibitory activity, the imbalance between the levels of MMPs and TIMPs is critical for ECM remodeling being able to provoke tissue destruction (18, 23). In this study, the linear regression analysis revealed a correlation of MMP-1 with both TIMP-1 ($\beta = 0.78$, $R^2 = 0.212$, $P = .041$) and TIMP-2 ($\beta = 1.83$, $R^2 = 0.231$, $P = .032$), which elucidates their association in apical periodontitis. It is worth pointing out that active MMPs are rapidly sequestered by TIMPs (38), and the enzyme-inhibitor complexes cannot be reactivated to any extent. TIMPs also bind to pro-MMPs and regulate MMP activation (ie, MMP-2, membrane type-1 [MT1]-MMP, and TIMP-2 complexes are required for activation of pro-MMP-2) (26). Detailed kinetic studies of the TIMP-MMP associations are reported in the literature (39).

Rather than calculating the ratio of MMPs and TIMPs, we sought to evaluate the formation of MMP/TIMP complexes. To the best of our knowledge, this is the first study investigating the bound form of MMP and TIMP in apical periodontitis. The search for the MMP/TIMP complex has been speculated by other research fields as a potential biological biomarker for the possible prediction of changes in the course of diseases (26, 27). In this study, MMP/TIMP complexes were detected in all apical periodontitis samples, with MMP-9/TIMP-1 being found at higher levels. No correlation was found in the MMP/TIMP complexes, with no clinical/radiographic feature indicating low or no value in such prediction. Furthermore, future studies should consider the MMP/TIMP complex measurements when comparing acute and chronic infections.

Because bacteria and their by-products (eg, endotoxins) are considered potential modulators of MMP and TIMP expression, we determined the levels of endotoxins present in root canal infection and correlated them with the levels of MMPs and TIMPs found in apical lesions. Endotoxins (ie, LPSs) were detected in all root canal samples from primarily infected root canals (31, 32). Higher levels of endotoxins present in root canals were positively correlated with larger amounts of MMP-9. Jotwani et al (40) revealed that LPSs are a relatively potent inducer of MMP-9.

Our findings have revealed that MMPs, TIMPs, and their complexes (MMP/TIMP) are involved in apical periodontitis by interacting with complex networks in the development of clinical features and the severity of bone destruction.

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

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